CORRELATION OF PERIPHERAL BLOOD FILM, RED CELL INDICES, BONE MARROW STUDY AND SERUM IRON STUDIES IN THE DIFFERENTIAL DIAGNOSIS OF MICROCYTIC HYPOCHROMIC ANEMIA IN CHILDREN

DISSERTATION

SUBMITTED FOR M.D. BRANCH III

[PATHOLOGY]

APRIL – 2016

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CHENNAI - TAMILNADU

CERTIFICATE

This is to certify that this dissertation entitled as **"CORRELATION OF PERIPHERAL BLOOD FILM, RED CELL INDICES, BONE MARROW STUDY AND SERUM IRON STUDIES IN THE DIFFERENTIAL DIAGNOSIS OF MICROCYTIC HYPOCHROMIC ANEMIA IN CHILDREN"** is the bona fide record work done by Dr.S.R. Murali Prasath submitted as partial fulfillment for the requirements of M.D. Degree Examination in Pathology to be held in April 2016.

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DECLARATION

I Dr. S.R. Murali Prasath solemnly declare that this Dissertation **"CORRELATION OF PERIPHERAL BLOOD FILM, RED CELL INDICES, BONE MARROW STUDY AND SERUM IRON STUDIES IN THE DIFFERENTIAL DIAGNOSIS OF MICROCYTIC HYPOCHROMIC ANEMIA IN CHILDREN"** is a bonafide record of work done by me in the Department of Pathology, Thanjavur Medical College and Hospital, Thanjavur under the Guidance and Supervision of my Professor **Dr.AL.SANTHI, M.D.,D.G.O**, The Head of the Department, Department of Pathology, Thanjavur Medical College, Thanjavur between 2013 and 2016.

This Dissertation is submitted to The Tamilnadu Dr. M.G.R Medical University , Chennai in partial fulfilment of University regulations for the award of M.D Degree (Branch – III) in Pathology to be held in April 2016.

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submitted by Dr. S.R. MURALI PRASATH **of**

Dept. of PATHOLOGY **Thanjavur Medical College, Thanjavur**

was approved by the Ethical Committee.

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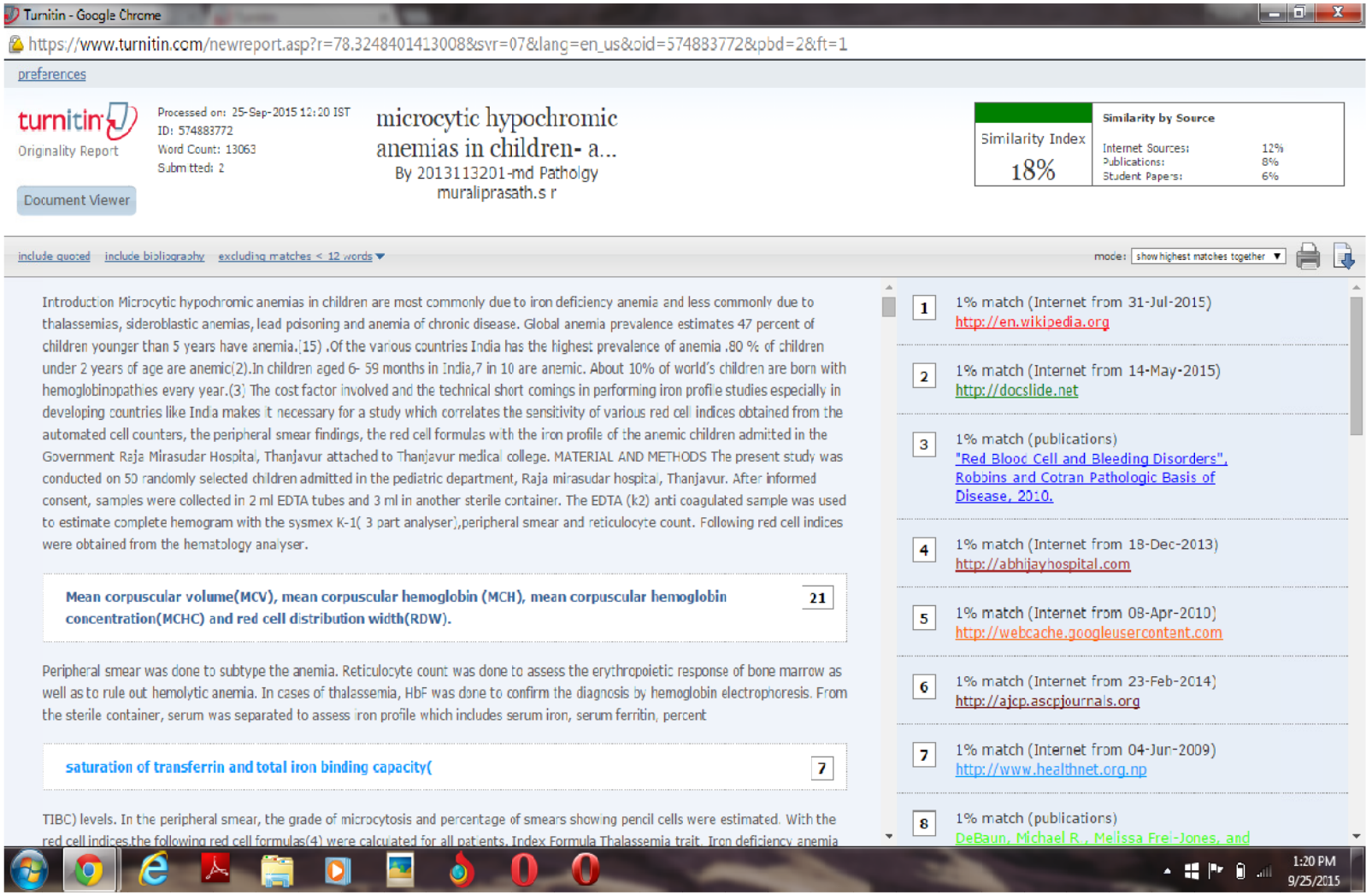
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Introduction Microcytic hypochromic anemias in children are most commonly due to iron deficiency anemia and less commonly due to thalassemias, sideroblastic anemias, lead poisoning and anemia of chronic disease. Global anemia prevalence estimates 47 percent of children younger than 5 years have anemia.[15) .Of the various countries India has the highest prevalence of anemia .80 % of children under 2 years of age are anemic(2).In children aged 6- 59 months in India,7 in 10 are anemic. About 10% of world's children are born with hemoglobinopathies every year.(3) The cost factor involved and the technical short comings in performing iron profile studies especially in developing countries like India makes it necessary for a study which correlates the sensitivity of various red cell indices obtained from the automated cell counters, the peripheral smear findings, the red cell formulas with the iron profile of the anemic children admitted in the Government Raja Mirasudar Hospital, Thanjavur attached to Thanjavur medical college. MATERIAL AND METHODS The present study was conducted on 50 randomly selected children admitted in the pediatric department, Raja mirasudar hospital, Thanjavur. After informed consent, samples were collected in 2 ml EDTA tubes and 3 ml in another sterile container. The EDTA (k2) anti coagulated sample was used to estimate complete hemogram with the sysmex K-1(3 part analyser),peripheral smear and reticulocyte count. Following red cell indices were obtained from the hematology analyser.
Mean corpuscular volume(MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration(MCHC) and red cell distribution width(RDW).
21
Peripheral smear was done to subtype the anemia. Reticulocyte count was done to assess the erythropoietic response of bone marrow as well as to rule out hemolytic anemia. In cases of thalassemia, HbF was done to confirm the diagnosis by hemoglobin electrophoresis. From the sterile container, serum was separated to assess iron profile which includes serum iron, serum ferritin, percent
saturation of transferrin and total iron binding capacity(
7
TIBC) levels. In the peripheral smear, the grade of microcytosis and percentage of smears showing pencil cells were estimated. With the red cell indices,the following red cell formulas(4) were calculated for all patients. Index Formula Thalassemia trait. Iron deficiency anemia
1 1% match (Internet from 31-Jul-2015) http://en.wikipedia.org
2 1% match (Internet from 14-May-2015) http://docslide.net
3 1% match (publications) "Red Blood Cell and Bleeding Disorders", Robbins and Cotran Pathologic Basis of Disease, 2010.
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CONTENTS

ABBREVIATIONS USED IN THE STUDY.

ACD- Anemia of Chronic Disease.

AHA- Autoimmune Hemolytic Anemia.

CBC - Complete Hemogram.

CHr- Reticulocyte Hemoglobin Concentration

CRP- C-Reactive Protein.

DMT-1- Divalent Metal Transporter1

EDTA- Ethylene DiamineTetra Acetic Acid.

FEP-Free Erythrocyte Protoporphyrin.

HBA- Adult Hemoglobin.

HBF- Fetal Hemoglobin

HBF- Fetal Hemoglobin

HCT- Hematocrit.

HPLC- High Performance Liquid Chromatography.

IDA- Iron Deficiency Anemia.

LDH- Lactate Dehydrogenase.

MCH- Mean Corpuscular Hemoglobin.

MCHC- Mean Corpuscular Hemoglobin Concentration.

MCHD- Mean Corpuscular Hemoglobin Density.

MCV- Mean Corpuscular Volume.

MDHL - Mean Density of Hemoglobin per Litre.

NPV- Negative Predictive Value.

PPV- Positive Predictive Value.

RDW- Red Cell Distribution Width.

RDW(CV)- Red Cell Distribution Width-Coefficient of variation.

RDW(SD)-Red Cell Distribution Width- Standard Deviation.

RDWI- Red Cell Distribution Width Index.

sTfR- Soluble Transferrin Receptor.

TIBC- Total Iron Binding Capacity.

WHO- World Health Organisation

β -TT- Beta Thalassemia Trait.

ABSTRACT :

Microcytic hypochromic anemia is the most common type of anemia in childhood. The differential diagnosis includes Iron deficiency anemia, anemia of chronic disease, thalassemia and sideroblastic anemia .Iron deficiency anemia is a major global health problem as it causes anemia as well as impaired coginitive and motor development along with behavioural abnormalities. In developing countries 39 % children below 5 years and 48 % children between 5 -14 years suffer from anemia. Children present in the hospital either with symptoms and signs of anemia or with manifestations of other diseases during which screening reveals anemia. In the current study, 50 children in the age group between 6 months to 12 years, with microcytic hypochromic anemia, identified in the peripheral smear report as well as complete blood hemogram, were randomly selected and their red cell indices were correlated with serum iron profile assuming them to be sensitive and specific for the differential diagnosis. The predominant age group involved was between 6 months to 5 years. Male to female ratio was 1.7:1. Mean age was 5.6 years. Out of 50 cases, 41(82%) were IDA cases, 8(16%) were anemia of chronic disease cases and only 1 (2%) was thalassemia major. In the differential diagnosis of microcytic hypochromic anemia, the correlative study revealed red cell distribution width (RDW) to be most sensitive in the diagnosis of iron deficiency anemia. There was also a statistically significant correlation between the RDW and serum iron profile. Of the various red cell formulas, Sirdah, RBC count, England &Fraser together were 100 % sensitive followed by RDWI and Mentzer's index, in the diagnosis of IDA. For the ACD cases, the cost effective approach is to correlate the clinical features and perform a CRP level to confirm the underlying chronic disease. After the treatment of underlying disease if the anemia is not corrected, then serum iron can be performed. In our study, all ACD cases had elevated CRP levels along with the clinical features

of underlying diseases. Also the RDW was normal in 6 out of 8 cases of ACD. For thalassemia major, owing to life long transfusion necessity, costly investigations like Hb electrophoresis are needed for confirmation.

KEY WORDS:

Microcytic Hypochromic anemia, Iron deficiency anemia, Anemia of chronic disease, Thalassemia major, Red Cell Distribution Width, Red Cell Formulas, Serum Iron Profile.

INTRODUCTION

Microcytic hypochromic anemias in children are most commonly due to iron deficiency anemia and less commonly due to thalassemias, sideroblastic anemias, lead poisoning and anemia of chronic disease. Global anemia prevalence estimates 47 percent of children younger than 5 years have anemia[15]. Of the various countries India has the highest prevalence of anemia .80 % of children under 2 years of age are anemic[2].In children aged 6-59 months in India,7 in 10 are anemic. About 10% of world's children are born with hemoglobinopathies every year.[3]

The cost factor involved and the technical short comings in performing iron profile studies especially in developing countries like India makes it necessary for a study which correlates the sensitivity of various red cell indices obtained from the automated cell counters, the peripheral smear findings, the red cell formulas with the iron profile of the anemic children admitted in the Government Raja Mirasudar Hospital, Thanjavur attached to Thanjavur Medical College.

MATERIAL AND METHODS

The present study was conducted on 50 randomly selected children admitted in the pediatric department, Raja Mirasudar hospital, Thanjavur. After informed consent, samples were collected in 2 ml EDTA tubes and 3 ml in another sterile container. The EDTA (k2) anti coagulated sample was used to estimate complete hemogram with the sysmex K-1(3 part analyser), peripheral smear and reticulocyte count.

Following red cell indices were obtained from the hematology analyser. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width(RDW). Peripheral smear was done to subtype the anemia. Reticulocyte count was done to assess the erythropoietic response of bone marrow as well as to rule out hemolytic anemia. In cases of thalassemia, HbF was done to confirm the diagnosis by hemoglobin electrophoresis. From the sterile container, serum was separated to assess iron profile which includes serum iron, serum ferritin, percent saturation of transferrin and total iron binding capacity (TIBC) levels.

In the peripheral smear, the grade of microcytosis and percentage of smears showing pencil cells were estimated.

With the red cell indices, the following red cell formulas[4] were calculated for all patients.

Index	Formula	Thalassemia trait.	Iron deficiency anemia
England &Fraser	MCV-RBC-(5XHB) -8.4	< 0 (neg)	>0 (pos)
Mentzer's	MCV/RBC	Less than 13	Greater than 13
Srivastava	MCH/RBC	<3.8	>3.8
Shine and lal	(MCV)(MCV)X MCH /100	<1530	>1530
Green and king	MCVXMCVXRDW/Hb x 100	<72	>72
Ricerca	RDW/RBC	<3.3	>3.3
RDWI	MCV x RDW/RBC	<220	>220
SIRDAH	MCV-RBC-(3X Hb)		

All the data compiled and tabulated for correlation. The parameters taken for correlation were:

1.Red cell indices and iron profile values.

2.Red cell formulas and iron profile values.

Inclusion criteria:

1.Children from age 6 months to 12 years.

2.Anemic children as per WHO cut off values.

3.Microcytic hypochromia as per peripheral smear and red cell indices.

Exclusion criteria:

1.New born children and age less than 6 months.

2.Children with macrocytic and normocytic anemias.

3.Smears with markedly elevated reticulocyte counts.

AIMS AND OBJECTIVES

1.To find out the proportion of various causes of microcytic hypochromic anemia.

2.To calculate the sensitivity and specificity values of red cell distribution width(RDW) and red cell formulas.

3.To correlate these values with iron profile values assuming them to be most sensitive.

4.To find out which red cell index identifies the cause of anemia without iron values(red cell index with highest sensitivity) especially red cell distribution width.

REVIEW OF LITERATURE

Worldwide 1.62 billion people are affected by anemia.Children less than five years show the highest prevalence. 48% of children between 5-14 years suffer from anemia.(WHO Data base 2005). In the World health organisation report it was estimated that 12% of children less than five years age were anemic in the developed countries and 51% of children in the same age group in the developing world are anemic.[9][43]

Anemia is defined as hemoglobin or hematocrit lower than age & sex adjusted range for healthy children.[1] Functionally anemia is defined as inadequate RBC mass to carry oxygen to the tissues.[6] Depending on age, gender and race normal values show variation. In anemia, the metabolic needs of the body are poorly met with. By itself it is not a disease.Pediatric reference ranges are helpful because analysers cannot detect normal age related variations in Hb,HCT,MCV etc. throughout childhood. Comparison with age specific values is necessary for all abnormal values. Any value less than 2 SD is regarded as abnormal.

Table 1[(1):]

Normal hemoglobin,hematocrit and mean corpuscular volume values in various ages.

	HEMOGLOBIN(g/dL)		**HEMATOCRIT(%)**		**MCV(μm^3)**	
Age(years)	Mean value	Lower limit	Mean	Lower limit	Mean value	Lower limit
0.5-1.9	12.5	11	37	33	77	70
2-4	12.5	11	38	34	79	73
5-7	13	11.5	39	35	81	75
8-11	13.5	12.0	40	36	83	76
12-14 Female male	 13.5 14.0	 12.0 12.5	 41 43	 36 37	 85 84	 78 77

Table 2:[23]

Hemoglobin concentration thresold used to define anemia as per WHO guidelines:

Age or Gender	Hb thresold level(g/l)	HCT
Children (6months to 5 years)	110	<33
Children (5 years to 12 years)	115	<34
Children (12 years to 15 years)	120	<36

WHO reports state when the anemia prevalence level is greater than 40, it becomes a severe public health problem. When the level is reduced to less than 4.9, it ceases to be a health problem.The corresponding mild and moderated levels of prevalence are 5 to 19.9(mild) and 20 to 39.9(moderate).

ERYTHROPOIESIS:

Erythropoiesis is the stepwise, programmed and regulated process during which the precursors called erythroblasts , otherwise called normoblasts (with normal morphology) undergo changes in the cytoplasm and nucleus resulting in decrease in the size. Gradually during the process of differentiation, the proliferative capacity decreases with hemoglobin becoming the predominant cytoplasmic protein.

ERYTHROID SERIES:

1.Proerythroblast:

This large cell measures 14 -20 micro metres in diameter. Being the least mature of the series, the cell membrane is round with the characteristic feature seen in the cytoplasm. The cytoplasm is basophilic compared to the myeloblast. Conspicuous round nucleoli are seen occcupying most of the cytoplasm.They are capable of rapid division.

2. Basophil erythroblast:

This round cell with a diameter 12 -16 µm has a more basophilic cytoplasm than it's precursor cell. It is also capable of rapid proliferation. The nucleus is larger and coarser with basophilic chromatin.

3.*Polychromatic erythroblast*:

The diameter is 12 – 14 µm. The characteristic feature is the polychromatic cytoplasm which is due to mixture of acidophilic hemoglobin and basophilic ribonucleic acid(RNA). Proliferation ceases after this stage. Nucleus becomes more coarser. This cell is also called intermediate eythroblast because this stage occupies an intermediate position between the previous stages and the later stages with the predominance of hemoglobin in the cytoplasm.

4.Orthochromatic erythroblasts:

In this final stage of maturation, the nucleus becomes small and pyknotic with a blue black appearance. But the cytoplasm is predominantly acidophilic due to hemoglobin content of the cytoplasm.

5.Reticulocyte:

When the nucleus is extricated from the cytoplasm, the orthochromatic normoblast becomes a reticulocyte. As the reticulocytes have greater diameter, when their numbers increases, the mean corpuscular volume also increases. As the reticulocytes lose their mitochondria and ribosomes, basophilia decreases to become a mature erythrocyte.

CLASSIFICATION: [1,6]

Anemia is classified on the basis of functional disturbances into

1.Disorders of effective red cell production.

2.Disorders of rapid erythrocyte destruction or red cell loss.

Anemia is classified morphologically on the basis of mean corpuscular volume into:

1.Microcytic hypochromic anemias.

2. Normocytic normochromic anemias.

3.Macrocytic Normochromic anemias.

Majority of the conditions that cause anemia also cause definable changes in mean corpuscular volume and can be grouped with this parameter.Anemia in children is caused by diseases that result in microcytic RBC'S.[2]

HEMOGLOBIN OVERVIEW:[1,2]

Microcytic anemia is caused by derangements in heme metabolism or globin synthesis. Hemoglobin is made up of heme ring with iron and 4 globin chains. Dominant hemoglobin in utero is HbF with 2 alpha and 2 gamma globin

chains.HbA,adult hemoglobin made of alpha and beta chains and HbA 2 with alpha and delta chains are dominant during intra uterine life. At birth 80 % hemoglobin is HbF and HbA is 20%. By 6 months of life HbF is gradually replaced by HbA.The concentration of hemoglobin at birth is 14g/dl which becomes 11g/dl by 1 year(physiological anemia of infancy).MCV at birth is 100 to 130fml.It decreases to70 to 85 fl by 1 year of age.

EVALUATION OF ANEMIA:[1,2]

It is important that laboratory tests must be interpreted in the context of history and clinical examination. Only minimum and important labortant tests need to be ordered.

Initial laboratory tests are hemoglobin estimation and hematocrit determination. Following which a peripheral smear and reticulocyte count is done. Others like platelet count and white cell count is sometimes needed.

Special investigations like measurment of erythrocyte porphyrin, serum ferritin,supravital staining,hemoglobin electrophoresis and finally a bone marrow examination is needed when indicated.

Evaluation of basic hematology laboratory data[6]:

To identify the cause of anemia the details obtained from the history and clinical examination must be correlated with the lab tests.Some useful questions to be asked are:

Is the anemia associated with other hematologic abnormalities?

If the answer is yes, then a bone marrow examination is performed to rule out leukemia, aplastic anemia, myelodysplasia and other marrow failure syndromes.

If the answer is no, then the next question to be asked is

Is the reticulocyte response to anemia is appropriate?

The reticulocyte count gives a clue if the bone marrow is adequately responsive or not and whether the RBC's are destroyed in the circulation or blood loss.

Traditionally reticulocyte count is measured by microscopic examination of a smear prepared from fresh blood stained with a supravital stain like new methylene blue.In the children the normal reticulocyte count is up to 5% in contrary to adults(0.5 to2%). The reticulocyte count needs to be corrected for the degree of anemia.

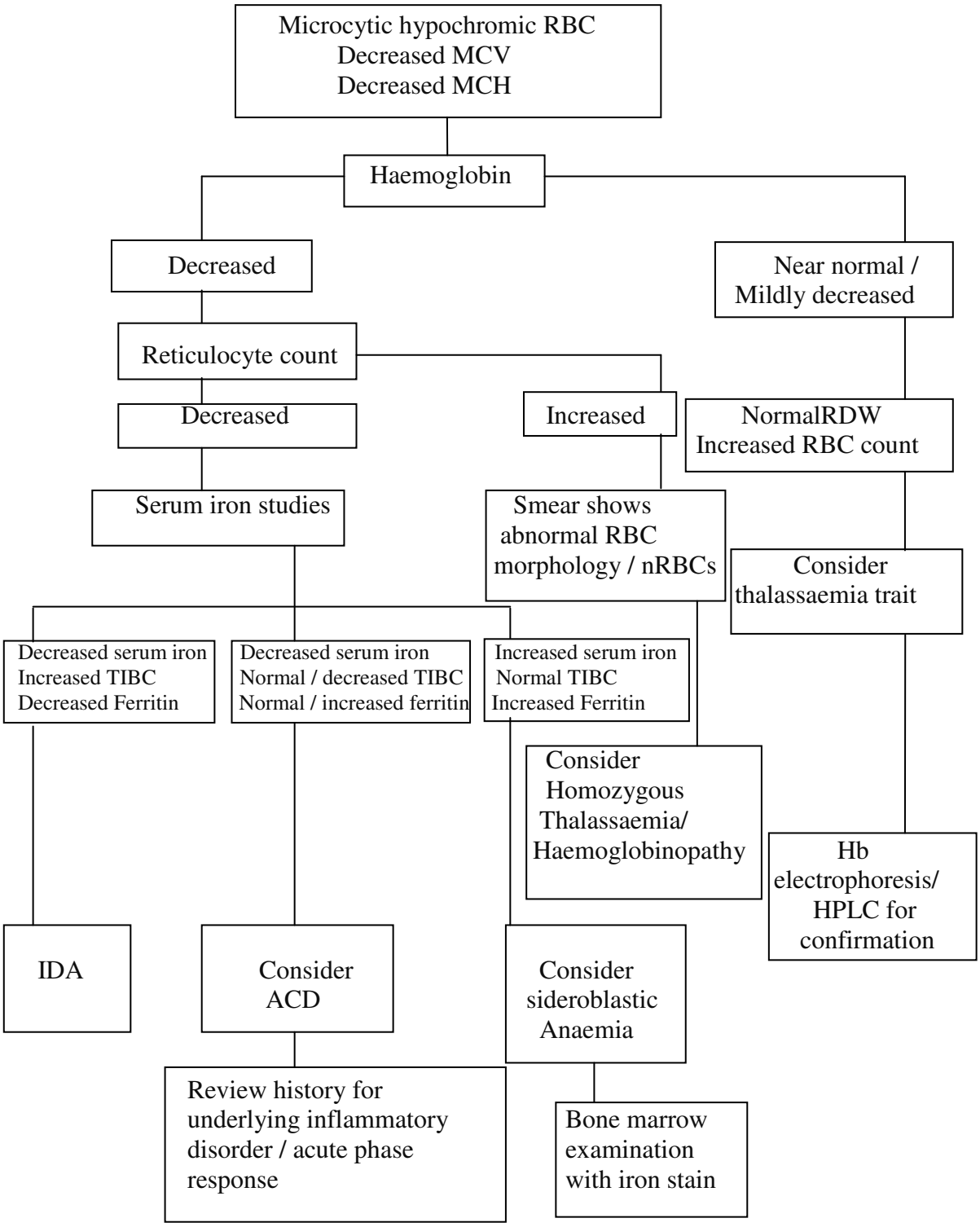
1.Reticulocyte count= % reticulocytes in RBC population.

2.Corrected reticulocyte count= % reticulocytes x (patient's hematocrit/45)

3. Absolute reticulocyte count= % reticulocytes x RBC count/L^3. normal values are from 25 to 75 $x10^9$/ L. values less than 100 indicate inappropriately low erythropoietic response to anemia.

If the answer to above question is yes then evidence of hemolysis such as increased bilirubin, increased LDH,decreased haptoglobin and hemosiderinuria must be sought. If the answer is no,then the red cell indices must be checked. If the MCV is less than 80 fml, microcytic anemia must be considered and evaluated.

APPROACH TO MICROCYTIC HYPOCHROMIC ANEMIAS:(DeGruchy's) [45]



Key history points:[1,6]:

Age:

In infants aged 6 months to 5 years iron deficiency is more common. Severe β thalassemia is diagnosed by age 6 months to 2 years.other thalassemias are usually diagnosed by 3 years.

Gender:

No gender difference is observed..

Race:

In blacks Hemoglobin S and C is seen more commonly while in whites Beta thalassemia is more common.

Ethnicity:

Thalassemias are encountered commonly in patients of mediterranean origin and south east asia.

Diet:

Enquiry regarding intake of iron, vitamin B12 and folic acid containing foods must be noted.Pica if present may point to iron deficiency.Geo or pagopahgia refers to craving for clay or ice.Intake of cow's milk and it's volume is important.

Blood loss: [25]

In older children blood loss is a possible cause. Occult bleeding may lead to chronic iron deficiency. Bleeding may be secondary to inflammatory colitis,peptic

ulcer, meckel's diverticulum etc. In developing countries hook worm infestation is an important cause.

Family history:

Anemia in other members of the family must be enquired as some causes of microcytic anemias are inherited[2]

AUTOMATED CELL COUNTING [22]:

Owing to the greater precision, reproducibility and capacity for completing a large number of measurements quickly,automated cell counting is preferred in the modern days. More over they are efficient,cost effective,lack inter observer variability and size distribution errors. Many variables can be estimated which is not possible manually. Automation is employed in several fields in hematology for

1.Cell counts(automated hematology analysers)

2.Diagnosis of hemoglobinopathies(high performance liquid chromatography)

3.Immunophenotyping and diagnosis of leukemia and lymphomas(flow cytometry)

4.Coagulation(coagulometers).

Automated hematology analysers:

There are 2 types of hematology analysers:

1.*Semiautomated analysers*: some manual steps need to be carried out such as dilution of blood.only small number of variables can be evaluated.

2.*Fully automated analysers*: no sample handling is required. Greater number of variables can be evaluated.

Components of cell counters:

1.*Hydraulics*: aspirating unit,dispensers,diluters,mixing chambers,aperture baths and hemoglobinometer.

2.*Pneumatics*: vacuums and pressures for operative valves.

3. *Electrical systems*: analysers and computing circuits.

Principles of analysers:

Electronic impedance:

In 1956,WALLACE COULTER introduced this principle of electronic impedance. This is based on observation that blood cells conduct electricity poorly. Two chambers filled with buffered electrolyte solution are separated by a small aperture and constant DC is applied. When a cell passes through the aperture it displaces a volume of diluent and increases the resistance thus producing a voltage pulse which is then displayed on the oscilloscope.

Optical light scatter:

The cells are made to pass in a single stream before a light source. A photo diode detects the scattered light and converts it to electrical signals. These signals are analysed and presented graphically.

Optical light scatter principle is used in 5 part differential count of WBC'S while impedance technology gives 3 part differential.

SYSMEX KX-21 HAEMATOLOGY ANALYSER:

The Sysmex KX-21 is multi-parameter automatic blood cell counter. It is meant *for in vitro diagnostic use* in clinical laboratories.

The KX-21 can process approximately 60 samples an hour and displays on the LCD screen the particle distribution curves of WBC, RBC, and platelets, along with data of 18 parameters,

KX-21 performs speedy and accurate analysis of 18 parameters in blood and detects the abnormal samples. Analysis data allows detecting those samples which are outside the tolerance and need further analysis.

The KX-21 uses three detector blocks and 2 kinds of reagents for blood analysis. WBC detector block measures the WBC count using the DC detection method. RBC detector block measures the RBC count and platelets also using the DC detection method.

Hemoglobin concentration is measured by the HGB detector block using the non cyanide hemoglobin method.

ANALYSIS PARAMETERS:

The following parameters can be analysed by the instrument.

1) Whole WBC count in 1 μL of whole blood

2) LYM%

Ratio (%) of lymphocytes (small cells) to whole WBC

3) MXD%

Ratio (%) of the summation of basophils, eosinophils and monocytes (middle cells) to whole WBC

4) NEUT%

Ratio (%) of neutrophils (large cells) to whole WBC

5) LYM#

Absolute count of lymphocytes (small cells) in 1 μL of whole blood

6) MXD#

Absolute count of the basophils, eosinophils and monocytes (middle cells) in 1 μL of whole blood.

7) NEUT#

Absolute neutrophil count in 1 μL of whole blood

8) RBC

RBC count in 1 μL of whole blood

9) HGB (Hemoglobin)

Volume (gram) of hemoglobin in 1 dL of whole blood

10) HCT (Hematocrit value)

Ratio (%) of whole RBC volume in whole blood

11) MCV (Mean RBC volume)

Mean RBC volume (fL) in whole blood, calculated by Hct/RBC.

12) MCH (Mean RBC hemoglobin)

Mean hemoglobin volume (pg) per RBC, which is calculated by Hb/RBC.

13) MCHC (Mean RBC hemoglobin concentration)

Mean hemoglobin concentration (g/dL), which is calculated by Hb/Hct.

14) RDW-CV (RBC distribution width in CV)

RBC distribution width (%) calculated from the points defining 68.26% of the entire area from the peak of the RBC particle distribution curve.

15) RDW-SD (RBC distribution width - SD)

The distribution width (fL) at the height of 20% from the bottom when the peak RBC particle distribution curve is taken as 100%.

16) PLT (Platelet)

Platelet count in 1 μL of whole blood

17) PDW (Platelet distribution width)

The distribution width (fL) at the height of 20% from the bottom with the peak of Platelet particle distribution curve taken as 100%.

18) MPV (Mean platelet volume)

Mean volume of platelet (fL)

19) P-LCR (Large platelet ratio)

Analysis principle:

WBC: DC detection method.

RBC: DC detection method.

HGB: Non-cyanide hemoglobin analysis method.

Whole blood mode:

Blood in the sample container is aspirated as a whole without any dilution for analysis.

Prediluted mode:

Dilution of blood sample (1:26 ratio) is done in the blood obtained from the ear lobe or finger prick before analysis is used.

Accuracy:

1) Whole blood mode:

WBC: ±3% or $\pm 0.2 \times 10_3/\mu L$ or less

RBC: ±2% or $\pm 0.03 \times 10_6/\mu L$ or less

PLT: ±5% or ±10 × 10_3/μL or less

2) Pre-diluted mode:

WBC: ±5% or ±0.3 × 10_3/μL or less

RBC: ±3% or ±0.05 × 10_6/μL or less

PLT: ±8% or ±15 × 10_3/μL or less

MCV and MCH obtained from the analyser enables classification of anemia.The red cell histogram shows the height at which RDW can be measured (20%).The red cell distrubution width RDW, is obtained from the red blood cell histogram. The RDW refers to anisocytosis which inturn refers to red cell size variation. In a normal patient,the histogram is virtually symmetrical. RDW can be expressed as co efficient of variation by the following formula.

RDW(CV) = 1SD/MCV x 100.

RDW may be normal in spite of markedly increased MCV value. In normal individuals it ranges from 11.5% to 14.5% but it may vary as a function of the model of the electronic counter used. Normal values for infants and children appear to range from 1.5% to 15%.

CLASSIFICATION OF ANEMIA BASED ON MICROCYTOSIS AND DISTRIBUTION WIDTH: [7][21]

MCV	RDW NORMAL	RDW HIGH
Microcytosis (MCV<80 fl)	Thalassemia trait, ACD, hemoglobinopathies	Iron deficiency HBH disease, ACD, thalassemia trait, fragmentation syndromes.
Normocytosis (80-100fl)	ACD,hereditary spherocytosis, hemoglobinopathies, acute bleeding	Partially treated iron or vitamin deficiency, sickle cell disease.
Macrocytosis (MCV>100 fl)	Aplastic anemia, myelodysplasias	B12 or folate deficiency, AHA,cold agglutinin disease, myelodysplasias, liver and thyroid disease, alcoholism.

The degree of marrow response aids in classifying anemias as:

1.Hyperproliferative anemias.

2.Normoproliferative anemias.

3.Hypoproliferative anemias.

Above approach is helpful in understanding the pathogenetic process. In hypoproliferative anemia,bone marrow is unable to respond to anemia with reticulocytosis. In normo or hyper proliferative anemia the marrow is able to respond by increased red cell production.

CAUSES OF MICROCYTIC ANEMIA: [2]

1.***Iron deficiency anemia***.

2.***Anemia of chronic disease***

3 thalassemias.

4.Lead poisoning.

5. Sideroblastic anemia

Other less common causes are lead toxicity and other hemoglobinopathies.

Traditionally microcytic anemias are classified on the basis of MCV <80 fl.The RDW is useful in distinguishing thalassemia from iron deficiency in that in thalassemias the RDW is lower and the red cell count is higher where as in iron deficiency the RDW is higher than normal compared to the degree of anemia.In anemia of chronic disease values are extremely variable with normocytic as well as microcytic(particularly in patients with renal disease). serum iron profile can be used to differentiate iron deficiency anemia and anemia of chronic disease without a bone marrow examination.

EVALUATION OF PERIPHERAL BLOOD SMEAR: [19]

Peripheral blood film examination is the single most useful procedure in the initial evaluation of anemic patient.

The most common reasons for a blood film to be performed for a complete blood count (CBC) are: [19]

1.***Quantitative abnormalities:***

When a measured parameter of the CBC falls outside the defined range, a blood film is made.The accepted ranges of various CBC parameters are published by the International society for Laboratory Hematology and the Clinical and Laboratory Standards Institute).Each institution must adjust these ranges to fit their own patient requirements.

2.***Qualitative abnormalities***:

A blood film is made when morphologic features of certain cells are detected and flagged as abnormal by the automated hemocytometer.

3.***Delta check***:

In this case,a number of CBC data points are stored in a data base on an individual patient from that patient's previous CBC samples.Knowing this individual's historical trends,if a quantitative abnormality occurs on a current CBC sample in a specific parameter,but the quantitative abnormality is not past the preset limits to trigger a blood film,still a blood film is made if the change is significant enough compared with the known historical trend of that individual patient.

4.***Request by the clinician***:

A CBC may be with in the limits,but the clinician still requests a blood film relevant to the case to look for any abnormalities.

BLOOD SAMPLE COLLECTION:

CBC samples are drawn in lavender tubes containing K2 ethylene diamine tetraacetic acid (EDTA). This allows the blood sample to remain in a liquid state for analysis by a hemocytometer and for a blood film to be done.compared to K2 EDTA,the morphology of samples anticoagulated with citrated and heparinized tubes is suboptimal.

SLIDE PREPARATION:

Blood films must be made within 12 hours of collection of K2 EDTA sample lest significant artifacts affect the quality of sample.A proper spreading of the film is essential.The slide must be 75 x 25 mm with 1 mm thickness.[44] One end of the slide must be frosted, although it is expensive, to facilitate labelling. Ideally, a large area of cells 1 cell thick should be obtained. A poorly spread blood film will obscure basic morphology of the cells. Accurate assessment will then be difficult. Manual making depends on the skill and experience of the operator. For anemic blood the angle of spread must be wider and for polycythemic blood it must be wider.

The blood film slide must be fixed immediately and not left unfixed for more than a few hours which allows preservation of good morphology and prevents the transmission of infectious diseases to the personnel in contact with the slide. Delayed fixations introduces morphological artifacts. Fixation is done with methanol. A miniscule amount of water in methanol induces hydration artifacts.

STAINING OF THE SLIDE:

The various cellular structures are differentiated by staining the slide. This contrasting is the ability to visually distinguish one structure from another. Various Romanowsky stains are in use such as Giemsa,May Grunwald,Wright's but all these a combination of azure B,a cationic dye that stains nucleic acids bue and eosin, an anionic dye which stains hemoglobin and eosinophil granles red/orange.

Various determinants of the staining quality are the azure B:eosin ratio,the pH of the buffer, contaminants,timing of the staining,age of the staining etc.

Initial evaluation must be done with low power to determine the adequacy of cell distribution and staining.A poor blood film preparation includes loss of central pallor in RBC's,polygonal shapes and artifactual spherocytes which show no variation in central pallor and are larger than normal red cells.

Subsequent examination must be done first under high power and then oil immersion. Grading should be done for size,staining intensity,variation in colour and abnormalities in shape.[1].

A cover slip can be mounted either temporarily with oil or permanently with mountant that is miscible with xylene. It is beneficial as it allows storage of the slide without dust and scratch damage. The mountant must be neutral and the slide must be stored in the dark.

The CBC report must be reviewed to assess the indexes reported and to look for any flags or concerns raised by the hemocytometer. Correlation between the morphologic features and the reported values can be done.

The blood film must be reviewed before the clinical history is seen in order to objectively comment on the changes seen without being biased by the clinical suspicion. Later reviewing the clinical history helps the reviewer to look for a low level change relevant to the case.

IDENTIFYING INFORMATION : [19]

A unique laboratory number or the patient's hospital number is an essential information to avoid jumbling of data.

MICROSCOPIC ASSESSMENT OF THE BLOOD FILM:

INITIAL SCREEN:

Initially low power view is used to scan the film to look for very high or low cell counts,malignant cells,dual population of red blood cells etc. Both the head and tail end are not suitable for routine examination. The zone of morphology is the best area for assessment[19]. as in this area the red cells do not show overlap and the central pallor is clearly visible.

ASSESSMENT OF THE RED BLOOD CELLS:

1.***Arrangement:***

Abnormalities include rouleaux,agglutination or ball like formations.

2. ***Size:***

Red blood cells are roughly 7.5 μm in diameter. The size of a mature small lymphocyte is used to assess the size of a normal red blood cell. Larger cells are called macrocytic and smaller cells microcytic. In general, hemocytometer indexes are more accurate. Sometimes,however the MCH/MCV indexes may not recognize a dual

population of red cells seen in partially treated or transfused iron deficiency,sideroblastic anemias and combined B12 and iron deficiency.

3. ***Chromaisia:***

Chromaisia refers to the central pallor in the red blood cells. The related index is the MCHC(mean corpuscular hemoglobin concentration). Normal red cell has a central pallor approximately 1/3 to ½ of the red blood cell diameter. Hypochromasia indicates increased central pallor as seen in iron deficient and thalassemic states. The border between the stained area of the red cell and central pallor area must demonstrate a gradual progression. A punched out artifact may be seen in hydration artifact(punched out artifact). Hyperchromasia is seen spherocytosis,irregularly contracted red cells or some macrocytes.

4.***Colour:***

The eosin component of the Romanowsky stain imparts a pink colour to the normal red cell.polychromasia refers to the bluish/purplish/grayish colour of the red cells with increased amount of reticulin. They are called reticulocytes. To accurately quantitate reticulocytes,a reticulocyte count performed either by flow cytometric or by manual staining technique is necessary.

5.***Shape:***

Normal red cells have a round and oval shape. There are many variations in shape and have clinical relevance to underlying medical process. Various terminologies used to describe red cells in this context include: elliptocytes, ovalocytes, tear drop cells,spherocytes,acanthocytes,echinocytes,blister cells,bite cells,target cells. Red cell fragments are smaller cells without central pallor. They

have sharp angulated edges and have variable sizes.eg.comet cells,triangular cell,helmet cell and schistocytes.

6.***Inclusion bodie***s:

Howell jolly bodies,pappenheimer bodies and basophilic stippling. Nucleated red cells have larger,round pyknotic dark purplish nuclei which appear as inclusion. Red blood cell inclusion in malaria may show thin bluish rings with 1 or 2 small red nuclei.

GRADING OF RED BLOOD CELL MORPHOLOGIC ABNORMALITIES:[3,19]

A four point grading scale from 1+ to 4+ tied to a percentage of red cell abnormality.International Society for Laboratory Hemtology guidelines suggest changes greater than or equal to 2+ are clinically relevant. Gene Gulati blood cell morphology grading guide which was published in 2009 is an useful guide for morphologic grading.

Table 1: The following grading criterion was applied[1]

Cell type	Occasional	1+	2+	3+	4+
Anisocytosis	Occasional	<2×	2-3×	3-4×	>4×
Poikilocytosis %	NA	<25	25-50	50-75	>75
Microcytosis %	NA	<25	25-50	50-75	>75
Size		>3/4×	½-3/4×	½-3/4×	<1/2×
Macrocytosis %	NA	<25	25-50	50-75	>75
Size		<2×	2-3×	3-4×	>4×
Hypochromia %	NA	<25	25-50	50-75	>75
Central pallor		0.4	0.5-0.6	0.6-0.7	>0.7
Target cells %	<5	5-10	10-30	30-60	>60
Tear drop cells %	<1	1-3	3-6	6-12	>12
Schistocytes %	<1	1-3	3-6	6-12	>12
Spherocytes %	<1	1-3	3-6	6-12	>12
Sickle cell %	<5	5-10	10-30	30-60	>60
Acanthocytes%	<5	5-10	10-30	30-60	>60
Echinocytes %	<10	10-25	25-50	50-75	>75
Elliptocytes %	<6	6-20	20-50	50-75	>75
Ovalocytes, %	<6	6-20	20-50	50-75	>75
Blister cells %	<1	1-5	5-10	10-15	>15
Stomatocytes %	<5	5-10	10-30	30-60	>60

The differentiation between thalassemia especially heterozygous thalassemia from other causes of microcytic hypochromic anemia like iron deficiency anemia is so important in a therapeutic point of view to avoid iron overload. A mild erythrocytosis (high RBC count) and marked microcytosis(low MCV) are characteristic of thalassemia trait. The RBC count is usually decreased in patients with iron deficiency and the MCV may be normal or decreased depending on whether the iron deficiency is acute or chronic. Several formulas have been developed to help define the 2 disordes.None are infallible but all can be useful. The cell count based indices especially the Mentzer index are easily available and reliable methods to detect β-TT from iron deficiency anemia.

IRON DEFICIENCY ANEMIA:[1,2]

EPIDEMIOLOGY OF IRON DEFICIENCY IN CHILDREN:[8]

World wide, iron deficiency and iron deficiency anemia affect a large population of people.The Fourth National Health and Nutritional Examination Survey (NHANES 1V) reports that iron deficiency without anemia affects 7% of toddlers aged 1 to 2 years,9% of adolescent girls (Looker, 2002)

PHYSIOLOGIC CHEMISTRY OF IRON:

The ability of iron to exist in 2 stable oxidation states: Fe 2+ or Fe 3+ is the key to its biological utility. Under physiological conditions,iron must be complexed to iron binding agents termed chelators.eg.transferrin in plasma. This is crucial to acquisition of iron from the environment and to it's transport and storage with in the body.

IRON PROTEIN COMPLEXES:

Heme is an iron protoporphyrin complex IX is stable and the functional properties of heme is determined by the nature of the associated protein or ligands. In hemoglobin,the globin histidine residue donates fifth electron and the sixth comes from the molecular oxygen. This configuration enables hemoglobin to transport oxygen safely throughout the body.

IRON ACQUISTION AND DISTRIBUTION: [1,20,21]

The total body iron content of a normal individual varies from 3 to 5 g in the adult.in the new born infant it is 0.5g. An average of 0.8 mg of iron must be absorbed each day during the first 15 year of life.More over loss of iron from shedding of

mucosal cells is to be compensated by intake of further smaller amount. Absorption of dietary iron is assumed to be about 10%. For optimal nutrition, diet containing 8-10 mg of iron daily is necessary.It varies depending on the sex and weight of the individual. Iron increases roughly in proportion to body weight.

IRON ABSORPTION:

Hemoglobin iron is approximately 60-70 % of total body iron.Break down of this hemoglobin releases iron into the circulation where it is bound to transferrin, iron binding protein.This is reutilised by the marrow erythroblasts for hemoglobin synthesis.

Tissue iron or storage iron: It is equally divided between the reticuloendothelial cells like spleen,liver and bone marrow and hepatic parenchymal cells and skeletal muscle.

FERRITIN:

It is the predominant form in the hepatocytes where as hemosiderin is mainly seen in reticuloendothelial cells. Although normally not visible microscopically it is seen as bluish granules in tissues stained by prussian blue reaction. Ferritin is made of apoferritin and trivalent iron. Iso ferritins are isomers of ferritins with varying proportions of acidic and basic subunits. Heart and red cell ferritin contain acidic(H subunit). Liver,spleen and serum ferritin are L-subunit rich[33]

HEMOSIDERIN:

This insoluble form of storage iron represents aging of ferritin molecule with partial denaturation of apoferritin and increase in iron content. In unstained tissues it appears as golden yellow granules and as blue granules in potassim ferrocyanide

method. Inspection of amount of hemosiderin in bone marrow is an important method of assessing body iron stores.

Plasma or transport iron:

Transferrin ,a β-globulin of molecular weight 88,000 synthesised in liver is the major transport protein which binds one or two atoms of ferric iron. Not only it carries iron from alimentary tract to tissue stores but also to erythroblasts. Transferrin receptors are found in reticulocytes and erythroblasts to which transferrin is bound and iron is released into the cells.

Serum transferrin is present in a concentration which enables it to combine with 44-80 μ mol of iron per litre. This is called *iron binding capacity of the serum.* Serum iron is normal subjects is about 20 μmol/l.The percentage of total iron binding protein to which iron is attached is called *percentage saturation of the iron binding protein.* Serum iron divided by total iron binding capacity which is expressed as a percentage gives percentage saturation. Normal value is about 33 percent that is about one third saturation.

An immunoradiometric assay for measuring serum ferritin concentration was established by Addison et al (1972)[(34)]. In normal individuals serum ferritin is stable and its concentration is related to body iron stores. It is also age and sex dependent. Levels in children are high at birth but rapidly fall and are low from 6 months to 15 years of age. In iron deficiency concentrations are less than 12 μg/l and is diagnostic of iron deficiency.

Methods for assessing iron stores: (Brittenham et al(1981) and Jacob & worwood (1984)[35,36]

1.quantitative phlebotomy.

2.liver biopsy.

3.bone marrow aspiration and biopsy.

4.urinary excretion of iron aster infusion of chelating agent serum iron,TIBC and percentage iron saturation.

5.erythrocyte protoporphyrin.

6.serum and red cell ferritin.

7.dual energy computed tomography.

8.magnetic susceptibility.

IRON ABSORPTION:

Absorption occurs predominantly in the proximal duodenum as the pH and redox potential are suitable at this site.The first part of the duodenum shows highest expression of the key proteins involved in the iron absorption. Active erythropoiesis and iron deficiency upregulates iron absorption and vice versa. The ferrous iron is rapidly converted to the insouble ferric form at physiological pH. The pH in the duodenum is lowered by the acid produced in the stomach thereby enhancing the solubility and uptake of iron. Heme iron is absorbed separately from inorganic iron also more efficiently thus making meat, an excellent source of iron. The dietary factors which influence iron absorption are vitamin c(ascorbate) and citrate which

increase iron intake by acting as weak chelators that increase the solubility of iron in the duodenum. Plant phytates, tannins inhibit iron absorption.

Of the various pathways the well characterised pathway is divalent metal transporter DMT-1 pathway(Conrad and Umbreit 2002,2005)[37]. Separate pathways exist for the absorption of heme and non heme iron.

Mucosal uptake:

DMT -1 otherwise known as Nramp 2 or DCT -1 transports all divalent metals including ferrous but not ferric iron in the mucosal iron. Duodenal cytochrome b reductase on the enterocyte brush border reduces ferric to ferrous iron and makes it a substrate for transport by DMT-1.

Mobilferrin-integrin –paraferritin pathway transports ferric iron unreduced.

Iron transfer from mucosal cell to the lamina propria:

In the mucosal cell iron is deposited as ferritin and if iron stores are adequate, the worn out mucosal cells are shed after 3 to 4 days. The proteins involved in the transport from the basolateral portion of the mucosal cell to transferrin in the plasma are ferroportin or MTP-1 and hephaestin,an iron oxidase(McKie et al 2000 and Aisen et al 2000)[38,39]. Hephaestin is a multicopper enzyme that oxidises ferrous to ferric iron for incorporation into transferrin that binds only the ferric form.

The amount of storage iron in the body and the overall rate of erythropoiesis decide the rate of iron absorption.Hepcidin, a negative iron metabolism regulatory hormone is liver derived plasma peptide(Leong & Lonnerdal 2004)[40]. Intestinal iron absorption varies inversely with liver hepcidin expression. Iron uptake in duodenum and iron release from macrophages is inhibited by hepcidin.

IRON CYCLE:

After absorption, iron is bound to transferrin and it is moved to erythroblasts of the marrow. In the marrow, iron is released from the transferrin and incorporated into hemoglobin. The mature erythrocytes are then released in to the circulation. After 120 days, the aged erythrocytes are phagocytosed by the resident macrophages in the reticulo-endothelial system where the iron is extracted fron the hemoglobin and returned to plasma. Here it binds to transferrin again completing the cycle. With each cycle,a small proportion of iron is transferred to storage sites and stored as hemosiderin or ferritin.

INTRACELLULAR IRON TRANSPORT:

The greatest iron content of the body is found in the erythroid precursor cells making about 60 to 80% of the total body iron. Approximaely 0.1 % of total body iron circulates in the plasma as an exchangeable pool. The function of transferrin is three fold :

1. Iron is rendered soluble under physiologic conditions.

2.Prevention of iron mediated free radical toxicity.

3.Transport of iron into the cells.

Thus the most important physiologic transporter of iron into the cells is transferrin to most parts of the body. The liver is the major site of synthesis and secretion of transferrin. Other sites include sertoli cells of testiss, oligodendrocytes of the central nervous system, lymphocytes , muscle cells and mammary cells .As serum transferrin cannot cross the blood brain barrier, some amount of local synthesis in these tissues provides transferrin to these organs for iron transport.

The sum of all iron binding sites on transferrin constitutes the total iron – binding capacity(TIBC) of plasma. As each transferrin molecule can bind two iron atoms, the TIBC is twice the concentration of transferrin on a molar basis. Normally only one third of transferrin is filled with iron.

IRON BALANCE;

Since 0.5 to 1mg of iron is lost every day to maintain a normal iron balance 1 mg of iron must be absorbed from the diet every day. 80% of iron present in a new born infant is accreted during the third trimester of pregnancy. So premature infants have deficient total body iron.(Rober D.Baker and Frank R.Greer etal).The Institute of medicine(IOM) suggests for infants from 7 to 12 months of age, the recommended dietary allowance is 11 mg/day. For toddler's 1-3 years of age,it is 7 mg/day.

ETIOLOGY OF IRON DEFICIENCY:

The interaction between iron intake, physiological iron requirements and the potential for blood loss determines the development of iron deficiency.

Inadequate iron intake:

Dietary sources:

Heme iron derieved from the animal tissues is more readily absorbed than non heme iron as the uptake is independent of gastric pH. In population which is dependent on vegetarian diets iron deficiency is thus the most common nutritional anemia. This is compounded by the chronic blood loss from parasitic infestations and malaria.

Consumption of cow's milk leads to iron deficiency through several mechanisms.

1.Low bioavailability of iron in cow's milk than human milk.

2.Replacement of iron –rich foods by cow's milk.

3.Cow's milk contains calcium and caesin which interferes with iron absorption and low grade chronic hemorrhage caused by irritation of the lining of GI tract by cow's milk proteins.

4.cow's milk induced gastrointestinal bleeding.[27]

Poor bioavailability:

As iron is insoluble in aqueous solution, gastric acidity is required for absorption.plant sources have iron which has low solubility and powerful iron chelators that bind the iron molecule.

IRON DEFICIENCY ANEMIA:

Iron is the most common single nutrient deficiency among children in the developing world.Iron deficiency anemia is the most common cause of anemia in children. The more common iron deficiency without anemia is also important in that it adversely affects neurodevelopment and behaviour.(Robert D Baker et al 2010). Most vulnerable are those between the ages 6 and 24 months of age.

Iron deficiency may cause skin,mucosal and gastrointestinal abnormalities,low weight for age,reduced capacity for work and reduced immune response. It also compromises physical,motor,psychological,behavioural,,cognitive and language development. (Maria Hadler and Sigulem 2002). There is a well established association between iron deficiency anemia and impaired neurocognitive function

which is independent of psychosocial and environmental factors (oski 1979)[8]. Also iron deficiency without anemia was also found to result in impaired neurocognitive function (Akman et al,2004)

PATHOGENESIS OF IRON DEFICIENCY ANEMIA: [6]

In anemia of iron deficiency, the following pathogenetic factors are described

1.As a consequence of reduced iron supply,hemoglobin synthesis is impaired.

2.Generalised defect in cellular proliferation.

3.Survival of erythroid precursors and erythrocytes is reduced when the anemia is severe.

As the transferrin saturation falls below 15%, the iron supply to bone marrow becomes inadequate to meet the requirements of hemoglobin production. Free erythrocyte protoporphyrin (FEP) levels are elevated secondary to imbalance in the protoporphyrin : iron ratio.

The cellular proliferation is restricted in iron deficiency inspite of relative erythroid hyperplasia because the degree of hyperplasia is low for the degree for the anemia. “Ineffective erythropoiesis” is also seen in iron deficiency resulting in rapid destruction of defective immature erythroid cells .

STAGES OF IRON DEFICIENCY: (1,2,17,20,)

PRELATENT:

- Reduction in iron stores without reduced serum iron levels.
- Detected by a low serum ferritin measurement.
- Hb normal, MCV normal,Transferrin saturation normal,serum ferritin decreased,marrow iron decreased.

LATENT:

- Reduction in serum iron concentration and increase in iron binding capacity.
- blood hemoglobin levels are normal.
- Hb normal,MCV normal,TIBC increased,serum ferritin decreased,transferrin saturation decreased,marrow iron absent.

IRON DEFICIENCY ANEMIA:

- Hemoglobin levels fall below the lower limit of normal.
- Hb decreased,MCV decreased,TIBC increased,transferrin saturation decreased,marrow iron absent.

	Stage 1(prelatent)	Stage 2(latent)	Stage 3(anemia)
Bone marrow iron	Reduced	Absent	Absent
Serum ferritin	Reduced	<12µg/l	<12µg/l
Transferrin saturation	Normal	<16%	<16%
FEP,Zinc protoporphyrin	Normal	Increased	Increased
Serum transferrin receptor	Normal	Increased	Increased
Reticulocyte Hb content	Normal	Decreased	Decreased
Hemoglobin	Normal	Normal	Reduced
Mean corpuscular volume	Normal	Normal	Reduced
Symptoms	Fatigue and malaise		Pallor,pica and epithelial changes.

CAUSES OF IRON DEFICIENCY ANEMIA:

In children increased physiological demand during the period of growth is the important cause due to progressive increase in number of red cells and also the amount of hemoglobin.

Nutritional deficiency due to inadequate diet is another important factor in infants and young children.

Tropical sprue and celiac disease in children may also be responsible for long standing impairment of iron absorption as does congenital abnormalities of gastrointestinal tract.

Maternal iron deficiency, multiple pregnancy ,GI bleeding,consumption of large quantities of cow's milk are other causes.

CLINICAL FEATURES:

In infants and young children pallor,irritability,listlessness and anorexia are very prominent features.pallor can be appreciated in the conjunctiva,gums and palm creases. In dark pigmented children nailbeds are ideal sites.

Growth impairment occurs in infancy which cannot recover to normal levels even with iron therapy.Dopamine and serotonin receptor levels in the central nervous system are reduced permanently thus affecting the neurological development. Reduced myelination of spinal cord is also seen.Growth hormone levels are related to serum transferrin levels affecting height and weight. Delayed cognitive development and neuropsychological effects are seen in iron deficiency without anemia[30,3132]

PICA:

Craving to eat unusual substances like dirt,clay,ice(pagophagia),laundry starch,salt,cardboard, or hair is a classic manifestation of iron deficiency and is usually cured by iron administration with in 1-14 days of iron administration (Callinan & Hare 1988).

IMMUNITY AND INFECTION:

In iron deficiency, the lymphocyte mediated immunity is defective. Moreover, bacterial killing is also impaired.(Brock and Mullero 2000).

PHYSICAL FINDINGS:

Many children are asympomatic and mostly detected by screening programmes.

LABORATORY FINDINGS :

Initially the tissue iron stores represented by marrow hemosiderin disappear. In the absence of inflammatory disease,serum ferritin provides relatively accurate estimate of body iron stores.Maria Hadler and Dirce et al suggest that serum ferritin is increased by two to four times as ferritin is a positive reactant in the acute phase (17). So ,measurement of C-Reactive protein (CRP) can be done to rule out infections and inflammations. A low serum ferritin is very specific and early indicator of iron deficiency.(1). In the presence of infectious process, serum ferritin level increases, but less than 50 µg. / litre. Serum ferritin is a most useful of all the serum tests. Low value invariably signifies iron deficiency. This test lacks sensitivity.

SERUM IRON : Decreased.

TIBC : Increased

PERCENTAGE SATURATION OF TRANSFERRIN :

Transferrin saturation= serum ironx100/TIBC. Normal 20 to 45% .In both children and adults value <5 % is diagnostic of iron deficiency(11).values <16% is suggestive of iron deficiency.

FREE ERYTHROCYTE PROTOPORPHYRIN:(FEP)

They increase when the iron availability becomes rate limiting for hemoglobin synthesis.

PERIPHERAL BLOOD FILM:

Anisocytosis is an important early sign in iron deficiency.The chief finding is hypochromia(increase in the central area of pallor more than 2/3 than normal).The

more severe the anemia,the more severe the hypochromia with greater percentage of erythrocytes affected.Microcytosis and poikilocytosis are also seen. Pencil shaped elongated elliptical forms are characteristic of iron deficiency.(6)

RED CELL INDICES:

1.Red cell distribution width (RDW)[14,11,20]:

Mean cell volume (MCV) and reticulocyte count(Demoyer et al 1989) are the two traditional principal criteria for the initial classification of anemic disorders.Anisocytosis as the coefficient of variation of red blood cell size was first quantified by Price jones(Judish et al 1966).More recently, the use of an analog computation techique with the new automated blood cell analyser is used to derieve RDW.(Bessman et al 1983).RDW is elevated in iron deficiency. RDW is the equivalent of anisocytosis observed in the peripheral smear(DeMoeyer et al 1989).Values greater than 15% proved highly sensitive(71 to 100 %) but non specific(50 %) for iron deficiency.[11]). It is the standard deviation of the erythrocyte cell size divided by the average erythrocyte cell size (MCV) and provides the percentage of erythrocytes outside the reference range.Visvanath et al 2001 and England et al 1976 have showed RDW was more sensitive in detecting iron deficiency anemia to the extent of 100% in mild degrees of anemia thus aiding the early diagnosis of iron deficiency anemia.Mc Cluer et al 1985 have also concluded in their study that RDW was 100% sensitive and was useful in early detection of early iron deficiency.Bessman et al 1983 ,Patton et al 1991 have showed that the early hematological manifestation in iron deficiency anemia.RDW is very useful in distinguishing from other causes of microcytic hypochromic anemias.

MCH and MCHC are reduced in long standing or severe disease.(6).

Other findings :

White blood cell counts are normal.platelets may show thrombocytosis due to increased erythropoeitin which shows homology with thrombopoietin.

BONE MARROW[6,20]

In iron deficient subjects, bone marrow iron is reduced or absent. The characteristic finding is the decrease or absence of stainable iron in bone marrow macrophages.(20).In bone marrow aspirates ,hemosiderin is seen as golden yellow refractile granules.When stained by prussian blue hemosiderin turns blue and this is used to grade the marrow.

GRADE	CRITERIA
0	No iron granules observed.
1+	Small granules seen with oil immersion
2+	Few small granules with low power lens
3+	Numerous small granules in marrow particles.
4+	Large granules in small clumps.
5+	Dense large clumps of granules.
6+	Very large deposits obscuring marrow details.

THALASSEMIAS: [20,21]

Thalassemias are inherited genetic disorders in globin chain production.β-Thalassemias are characterised by complete absence of β-globin chain (β 0 thalassemia) or a partial reduction (β + thalassemia).α thalassemias are characterised by absent or partially reduced α globin chain production. The quantity of globin gene production is the primary pathology in thalassemia.

Originally thalassemias were described in Italians,Greeks and other people of mediterranean origin.But now it is seen in the Middle east,India,Southeast Asia and Blacks.Colah et al 2011 have reported that nearly 7500 to 12000 β thalassemia major babies are born in India each year.

HEMOGLOBIN TYPES:[22]

Hemoglobin A(Hb-A):

This comprises of 97 % of hemoglobin of adult red cells.consists of 2 α and 2 β chains with the structural formula α2β2.The α chain has 141 amino acids and the β chain has 146 amino acids.During the first few months of postnatal life,Hb-A almost completely replaces Hb-F and the adult pattern is fully established by 6 months.

HEMOGLOBIN A2(Hb-A2):

The structural formula is α2δ2 the delta chain containing 146 aminoacids. It is present in small amounts at birth and reaches adult levels or 1.5 to 3.2 percent during the first year of life.Elevation of Hb-A2 is a feature of some types of thalassemia.It may be reduced in iron deficiency.

HEMOGLOBIN F(Hb-F);

The structural formula is α2γ2. Each gamma chain contains 146 aminoacids.At term Hb-F accounts for 70-90 percent of the total hemoglobin. It then falls rapidly to 25 % at 1 month,and 5 % at 6 months.

Other hemoglobins are Hb –Gower 1 &2,embryonic hemoglobins.

PATHOPHYSIOLOGY:

Inadequate beta globin gene production leading to decreased levels of normal hemoglobin Hb-A and an imbalance in alpha and beta globin chain production are the 2 major features seen in thalassemia.In the bone marrow,thalassemic mutations disrupt the maturation of red cells leading to ineffective erythropoiesis.The marrow is hyperactive but the patient has relatively few reticulocytes and severe anemia.

In beta thalassemias,the excess alpha chains form alpha 4 tetramers.These inclusions interact with the red cell membrane and shorten rred cell survival causing anemia and incrresed erythroid production. The gamma globin chains are produced in increased amounts producing elevated HbF.

In alpha thalassemias there is excess of beta and gamma globin chains.These excess chains form Barts hemoglobin in fetal life and HbH (beta 4) at birth.These abnormal tetramers cause extravascular hemolysis.

BETA THALASSEMIA MAJOR:[12,20,21]

The disorder is a homozygous state for the beta thalassemia gene. Unless blood transfusions are given, death occurs during childhood .

MOLECULAR PATHOGENESIS:

The causative mutations fall into two categories:

(1) β^0 *mutations*, characterized by absent β-globin synthesis.

(2) β^+ *mutations*, characterized by reduced (but detectable) β-globin synthesis.

More than 100 different causative mutations, mostly consisting of point mutations have been identified.

- ***Splicing mutations***.

  These are the most common mutations which are seen in the introns leading to β^0-thalassemia as these mutations are capable of destroying the normal RNA splice junctions. Normal mRNA production is thus impaired. Mutations creating "ectopic" splice site within an intron leads to β^+-thalassemia . Both normal and abnormal splicing occurs resulting in the production of some normal β-globin mRNA.

- ***Promoter region mutations***.

  β^+-thalassemia is mostly found in these type of mutations as transcription is significantly reduced with synthesis of normal globin chains..

- ***Chain terminator mutations***.

  These are the most common cause of β^0-thalassemia.There are two subtypes of mutations in this category.. The most common type creates a new stop codon within an exon; the second introduces small insertions or deletions that shift the mRNA reading frames (frameshift mutations). Both block translation and prevent the synthesis of any functional β-globin.

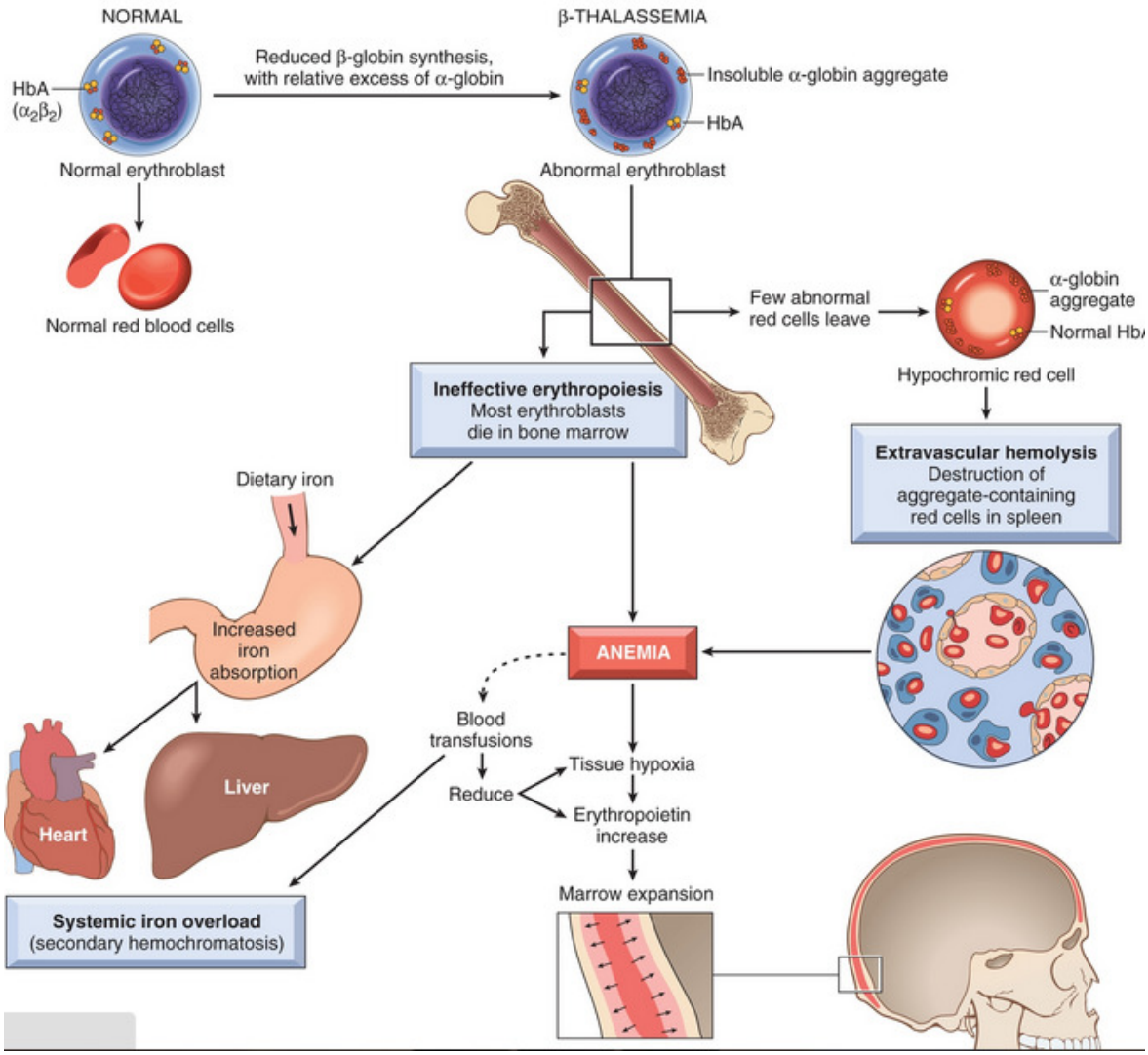
  The IVS-1-5 mutation is the commonest mutation found in the Indian population and its prevalence (in homozygous state) varies from 22.8 to 81.4% in different regions of India, being the highest in Tamil Nadu in southeastern India. In the north-western part of India the 619 bp deletion mutation is the commonest beta-thalassemia mutation

observed in patients originating from Sindh, Gujarat or among the families migrated from Pakistan during partition of the country in 1947.[25]

Impaired β-globin synthesis results in anemia by two mechanisms .

1. The defective HbA synthesis leads to "poorly hemoglobinized" microcytic and hypochromic RBCs.

2. The diminished survival of red cells and their precursors is due to insoluble inclusions of unpaired α chains, which precipitate within the red cell precursors. This results in membrane damage and subsequent apoptosis. Those red cells that are released from the marrow also bear inclusions and membrane damage and are prone to splenic sequestration and *extravascular hemolysis.* In severe β-thalassemia, it is estimated that 70% to 85% of red cell precursors suffer this fate, which leads to *ineffective erythropoiesis*. The first response to ineffective erythropoiesis and anaemia is an increased production of erythropoietin, causing a marked erythroid hyperplasia, which, in turn, may produce skeletal deformities, osteoporosis, and occasionally extramedullary masses, and contributes to splenomegaly. Untreated or undertreated thalassaemia major patients have retarded growth as a result of anaemia and the excessive metabolic burden imposed by erythroid expansion. Anaemia may produce cardiac enlargement and sometimes severe cardiacfailure. Ineffective erythropoiesis is also associated with increased iron absorption, which occurs mainly from increased intestinal absorption of iron caused by deficiency of hepcidin,, a 25-amino acid peptide produced by hepatocytes that plays a central role in the regulation of iron homeostasis.(42)



CLINICAL FEATURES:

Anemia:

The earliest symptoms of the disease is usually seen in the first year of life when the γ- chain synthesis is not replaced by corresponding increase in the synthesis of β- chains. The age at attending physician. The first sign is usually pallor with varying severity of splenomegaly and failure to thrive.[6] Non specific symptoms like anorexia and diarrhoea may also be seen. By the age of 3 years,splenomegaly

becomes obvious. Moderate to marked hepatomegaly is also seen.Mild jaundice is also seen.

Bone deformities:

In untransfused patients or poorly transfused patients, typical bone deformities are due to increased erythropoiesis and expansion of bone marrow to about 30 times than normal. The skull becomes large with anterior and posterior bossing with increased thickness of the diploe. There is thinning of the outer and inner tables of the skull with the trabeculae arranged in vertical striations giving rise to "hair on end" appearance. Zygomatic bones become prominent with depression of base of the nose. Maxillary overgrowth results in severe malocclusion simulating a rodent. The increased erythropoiesis is noticed first in the metatarsal and metacarpal bones as expansion which is useful indicator to predict the time for initiation of transfusion therapy. The ribs are broad giving a "rib within rib" appearance. The vertebral bodies give a squared out appearance. Premature fusion of the humeral and femoral epiphysis leads to shortening of long bones.

LABORATORY FINDINGS:

Anemia is usually severe.hemoglobin levels range between 3-9 gm/dl. Marked anisopoikilocytosis is common. Tear drop cells are seen before splenectomy. There is striking hypochromia as also target cells. Nucleated RBC's are also seen. Basophilic stippling is characteristically seen.

MCV and MCH are significantly reduced.

Polychromasia and punctate basophilia are seen to a moderate degree.

Reticulocyte count is elevated only in patients who have undergone splenectomy.

Osmotic fragility test shows an increased resistance to hemolysis.

Serum bilirubin is moderately raised.

Serum iron and ferritin:elevated.

Serum transferrin: fully saturated.

Examination of siblings, parents and children is extremely important.

BONE MARROW ASPIRATION:

Hyperplastic erythropoiesis with increased proportion of basophilic and polychromatic normoblasts with smaller cytoplasm. Some normoblasts show methyl violet positive inclusions. Siderotic granules are seen scattered throughout cytoplasm.

HEMOGLOBIN PATTERN:

Predominantly Hb-F is seen with 10-98 percent of total. Hb-A is completely absent with variable amounts of Hb-A 2. It may be up to 7 %. The remaining portion is contributed by HbA.

ANEMIA OF CHRONIC DISEASE:[20,22]

First described by Cartwright half century ago. This type of anemia is associated with number of chronic systemic diseases associated with infection,inflammation, or tissue break down. Infections include bronchiectasis and osteomyelitis. Inflammatory conditions are rheumatoid arthritis,SLE and ulcerative colitis.

PATHOGENESIS:

Decreased RBC life span, relatively failure of bone marrow to respond to anemia and decreased iron availability. Also the inflammatory cytokines chiefly IL-6 induces the production of *hepcidin* which decreases intestinal iron absorption and blocks release of iron from macrophages. In response to inflammatory cytokines, increasingly IL-6,[24] the liver produces increased amounts of hepcidin. Hepcidin in turn causes increased internalisation of ferroportin molecules on cell membranes which prevents release from iron stores. Inflammatory cytokines also appear to affect other important elements of iron metabolism, including decreasing ferroportin expression, and probably directly blunting erythropoiesis by decreasing the ability of the bone marrow to respond to erythropoietin.

In addition to effects of iron sequestration, inflammatory cytokines promote the production of white blood cells. Bone marrow produces both white blood cells and red blood cells from the same precursor stem cells. Therefore, the upregulation of white blood cells causes fewer stem cells to differentiate into red blood cells. This effect may be an important additional cause for the decreased erythropoiesis and red blood cell production seen in anemia of inflammation, even when erythropoietin levels are normal, and even aside from the effects of hepcidin. Nonetheless, there are other mechanisms that also contribute to the lowering of hemoglobin levels during inflammation: (i) Inflammatory cytokines suppress the proliferation of erythroid precursors in the bone marrow; (ii) inflammatory cytokines inhibit the release of erythropoietin (EPO) from the kidney; and (iii) the survival of circulating red cells is shortened.

CLINICAL FEATURES:

Most symptoms and signs are related to the underlying disorder with mild to moderate anemia.

LABORATORY FINDINGS:

Hemoglobin levels are 6-9gm/dl.

Usually anemia is normochromic and normocytic with modest microcytic,hypochromic features. Mild anisocytosis may be seen.

Reticulocyte counts are normal or low.

Serum iron: decreased.

TIBC: normal to low.

This pattern of low serum iron and low to normal iron binding protein is a regular and a valuable diagnostic feature.

Serum ferritin: elevated.

Bone marrow: normal cellularity.Iron content may be increased.

C-Reactive protein levels are elevated.In infants and children a level greater than 6 mg/l indicates infection or inflammation. This is an acute phase reactant whose concentration increases rapidly in infections and inflammations.[17] Inflammation is the key to the diagnosis and control of inflammation eliminates anemia.

SIDEROBLASTIC ANEMIAS:.

These are acquired and hereditary disorders of heme synthesis.Impaired heme synthesis leads to retention of iron with in the mitochondria. Only few sideroblastic anemias are seen in children.

CONGENITAL SIDEROBLASTIC ANEMIA:

This is a rare X linked congenital disorder.autosomal dominant pattern as well as sporadic forms are seen. The rate limiting enzyme 5-aminolevulinic acid synthetase is abnormal in all cases.

CLINICAL FEATURES:

Only severe anemias are seen in childhood with pallor,icterus and moderate splenomegaly and hepatomegaly.

LABORATORY FEATURES:

Microcytic hypochromic peripheral smear with normal RBC"s give a dimorphic picture.There is an extremely high RBC distribution width(RDW).

Serum iron: elevated.

Transferrin saturation: increased.

BONE MARROW:

Ringed sideroblasts which are nucleated RBC's with iron aggregates in mitochondria that have a perinuclear distrubution are seen.

DIFFERENTIAL DIAGNOSIS OF MICROCYTIC HYPOCHROMIC ANAEMIA – LABORATORY FEATURES (20)

Tests	Iron Deficiency	ACD	Thalassemia	Sideroblastic Anaemia
MCV	Decreased	Normal or Decreased	Markedly Decreased	Decreased
RDW	Increased	Increased or Normal	Increased	Increased or Normal
RBC Morphology				
Red cell count	Decreased	Decreased	Normal	Normal
Serum Iron	Decreased	Decreased or Normal	Increased or Normal	Increased or Normal
Tests	Iron Deficiency	ACD	Thalassemia	Sideroblastic Anaemia
TIBC	Increased	Decreased	Normal	Normal
Percent Saturation	Decreased	Decreased or Normal	Increased or Normal	Increased or Normal
Ferritin	Decreased	Increased or Normal	Normal	Normal
Serum Transferrin Receptor	Increased	Normal	Increased	Normal
FEP	Increased	Increased	Normal	Increased
Hb Electro Phoresis	Normal	Normal	Abnormal	Normal
Marrow Iron	Low or Absent	Increased or Normal	Normal	Ring Sideroblasts > 15%

CURRENT MODALITIES IN THE DIAGNOSIS OF MICROCYTIC ANAEMIA:

Soluble transferrin receptors: ([1,2,11,26])

It is an normal proteolytic cleavage product of transferrin receptor derieved from erythroid precursor cells.During the early development of iron deficiency serum transferrin receptor levels are increased and is a sensitive response.It is not affected by infection or inflammation.does not vary with age ,sex or pregnancy. But in ineffective erythropoiesis it may be elevated.In distinguishing iron deficiency from anemia of inflammation this parameter is very helpful.(oliveras et al 2000,Vazquez et al 2006).(8). TfR1 detects iron at cellular level. It is found on cell membranes. In deficiency states, there is upregulation of receptors and more concentrations are found in the serum. The cellular transferrin receptor (TfR) molecule is a transmembrane protein that binds transferrin, the principal iron transport protein found in the blood.1,2 TfR is found in highest concentration on the surface of cells requiring large amounts of iron, such as hemoglobin-synthesizing cells of the reticuloendothelial system (ie, bone marrow, liver, and spleen)3 and the placenta.4-7 In 1986, Kohgo et al, were the first to demonstrate the presence of a soluble form of TfR in human serum that was identified subsequently as a truncated form of cellular TfR derived from proteolytic cleavage of its extracellular segment.9-11 Moreover, 80% of the total serum level of TfR originates from immature RBC progenitors, including reticulocytes, while 20% originates from nonerythroid tissues.

***CHr*:[(1)(2)]**

Reticulocyte hemoglobin concentration[(29)] is available only in selected laboratories as it is unique to certain types of analysers. In a recent study it was found to have high and specificity in the detection of early iron deficiency.Both CHr and

TfR1 concentrations are not affected by inflammation and infection or anemia of chronic disease.CHr assay is currently available for use in children as it is validated and standard values have been determined. It provides a measure of iron available to cells recently released from the bone marrow. This is measured by flow cytometry

***TfR –index*:**

Ratio of soluble transferrin receptor to the log of serum ferritin values >15 suggest iron deficiency alone or in combination with inflammatory conditions. Value <15 is characteristic or anemia of chronic diseases. This index is also sensitive enough in detecting iron deficiency.(11)

Free erythrocyte protoporphyrin:

This parameter is useful in microcytic anemia due to lead poisoning as lead directly inhibits ferrochelatase the last enzyme in heme biosynthesis.

Ret Y:

It is a sensitive indicator of early iron deficiency.(Brunagara et al). Present automated hematology analyser can measure the reticulocyte size (Ret Y) values and correlates closely to sTfR. The sensitivity and specificity overall is highest of all the panel of tests.

In a prospective study it was found that a cut off of 27.5 pg had 83% sensitivity and 72% specificity.(1).

Hemoglobin separation to determine Hb pattern is performed in case of thalassemias with different methods like electrophoresis at alkaline pH,isoelectric focussing,high performance liquid chromatography.

MASTER CHART I

	perpheral smear no.	Age	Sex	IP No.	WBC (10^2)	RBC (10^6)	HGB (G/DL)	HCT(%)	MCV (FML)	MCH (pg)	MCH C (g/dl)	PLT (10^3/ µL)	RDW (CV)%	s.ferritin	TIBC	transferrin	%iron saturation	s.iron	DIAGNOSIS	CRP
1	ps 2124/14	3	F	311677	6.6	2	3.8	13.9	69.5	19	27.3	248	not mea	726.2	134	102	8.2	11	thal	
2	ps 2210/14	8	M	314631	9.5	5.06	7.7	39.2	57.7	15.3	26.4	958	22	12.4	154	121	10.3	16	ACD	57.8
3	ps 2096/14	4	M	311801	7	3.46	8.9	28	70.9	25.7	31.8	104	22.6	1256	135	106	12.5	17	ACD	85.2
4	ps 3222/14	12	M	315618	9.9	3.72	10.4	32.7	67.9	23	25.8	512	18	6	402	142	2.48	10	IDA	
5	ps 3310/14	10 mo	M	315973	9.4	4	8	29	74	22	29	428	20.8	71	41	32	31.7	13	IDA	
6	ps 3412/14	7	F	317117	9.1	4.04	7.3	29.2	72.3	18.1	25.6	282	24.3	9.6	130	102	12.3	16	IDA	
7	ps 3509/14	1	M	317873	9	3.5	4.4	20.1	56.6	12.4	21.9	408	29.8	4.2	124	97	14.5	18	IDA	
8	ps 3514/14	2	M	323683	16.1	4.19	5.8	20.1	56.8	13.7	23.9	300	28	8	310	80	7.09	22	IDA	
9	ps 3520/14	5	M	324765	5.8	2.63	4.8	16.8	63.9	18.3	27	186	30	20	502	220	9.07	49	IDA	
10	ps 3544/14	1	F	337826	15.2	3.29	6.3	21.7	66	19.1	29	266	23.5	5	420	200	10	42	IDA	
11	ps 3569/14	8	F	344535	5.2	3.29	5.9	19.8	60.2	17.9	29.8	179	34.9	10	612	176	1.96	12	IDA	
12	ps 4906/14	4.5	F	344875	15.1	4.27	9.5	33.2	67	22.2	28.2	585	18.8	7	392	201	7.40	29	IDA	
13	ps 4907/14	3	M	344856	10.3	4.15	9.8	32.5	70.3	23.6	30.2	315	17.9	8.2	456	272	0.88	4	IDA	
14	ps 4950/14	1	M	344978	6.7	3.91	8.3	31.2	79.8	21.2	26.6	317	19.7	11	411	126	6.33	26	IDA	
15	ps 4978/14	9	M	345090	10.7	4.18	7.5	28.8	68.9	17.9	26	785	13.3	102	98	172	11.22	11	ACD	99.5
16	ps 5708/14	6	F	346246	7.5	4.04	8.09	24.5	60.6	19.8	32.7	676	17.4	6.8	433	199	0.69	3	IDA	
17	ps 5810/14	6 mon	M	348420	8.9	3.58	8.1	29.1	71.3	22.6	27.8	765	16.5	5.8	421	214	9.03	38	IDA	
18	ps 245/15	2	F	348348	10.7	4.08	8.7	30.8	70	21.3	28.2	318	17.2	2	502	180	8.37	42	IDA	
19	ps 364/15	4	M	349176	8.5	3.57	4.1	19.8	55.5	11.5	20.7	602	29.7	7.2	410	252	9.51	39	IDA	

20	ps 1121/15	10	F	358165	12	3.35	6.5	23	68.7	19.4	28.3	115	12.9	102	152	199	23.03	35	ACD	101.7
21	ps 1355/15	2.5	M	356516	7.9	4.58	11.4	32.8	71.6	24.9	34.8	420	13.2	7	225	278	18.22	41	ACD	86.3
22	ps 1351/15	6	F	356511	10.3	4.68	10	29	76.7	20	35.9	511	12.7	7	410	102	7.80	32	IDA	
23	ps 1444/15	2	M	357474	10.5	4.64	6.3	25.2	54.3	13.6	25	404	22.2	3.2	425	265	3.11	13.2	IDA	
24	ps 1524/15	1.1	M	357713	9	4.63	7.8	28.3	61.1	16.8	27.6	511	23.1	10	396	120	11.36	45	IDA	
25	ps 1698/15	2.5	M	357608	12	4.75	9	28.1	59.2	18.9	32	393	18.8	15	629	222	1.43	9	IDA	
26	ps 1549/15	3	M	358584	15	4.25	10.9	32.9	77.4	25.6	33.1	515	15.5	20	403	175	12.41	50	IDA	
27	ps 1598/15	4.5	M	361574	6.9	2.1	6	15.5	73.8	28.6	38.7	182	26.6	3	712	282	0.84	6	IDA	
28	ps 2020/15	7	M	362960	21.6	3.29	8.6	25.4	77.2	25	30	569	19.9	10	430	192	8.84	38	IDA	
29	ps 2121/15	5	M	363546	7.5	2.99	4	17.5	58.5	22.9	33.4	269	27.6	7	496	37.1	6.05	30	IDA	
30	ps 2183/15	1.1	F	364529	15.3	3.95	11.4	31.9	80	25	27	402	12.3	140	104	190	21.15	22	ACD	55.8
31	ps 2162/15	1.2	M	369307	4.5	1.91	4.5	15.3	80	23.6	29.4	264	22.3	6	502	177	4.18	21	IDA	
32	ps 2175/15	9	F	374207	10.3	3.14	3.1	15.8	50.3	22	19.6	350	21.5	2	492	265	2.44	12	IDA	
33	ps 2422/15	5	F	372020	10.5	4.72	10	28	75	24.8	33.1	359	15.7	10	452	180	5.09	23	IDA	
34	ps 2427/15	3	F	372875	5.5	1.6	4.4	13.4	63.8	27.5	32.2	150	25.3	2.5	412	185	3.89	16	IDA	
35	ps 2432/15	2	M	372300	10.8	3.57	7.3	24.9	69.7	20.4	29.3	238	15.9	6.8	405	192.5	11.65	47.2	IDA	
36	ps 2449/15	6	M	372261	8.2	3.8	8.3	25.3	66.6	21.8	32.8	151	17.5	5.4	433	248.7	4.50	19.5	IDA	
37	ps 2461/15	10	F	371256	14.9	1.58	2.5	9.7	61.4	15.8	29.8	290	27.1	1.8	473	227.9	1.80	8.5	IDA	
38	ps 2513/15	1.5	M	372147	11.1	3.66	7.2	24.2	66.1	19.7	27.8	491	19.8	3.9	444	162.9	4.33	19.2	IDA	
39	ps 2527/15	1	M	323062	8	4.51	8.2	27.9	61	18.2	29.4	218	20.8	4.9	409	232.6	3.08	12.6	IDA	
40	ps 2528/15	9	M	373033	8.3	3.47	7.9	22.8	72.6	31.3	32	257	13.6	145	249	272.9	4.30	10.7	ACD	62
41	ps 2565/15	10	F	373423	8.8	4.5	9.9	31.3	75.4	23.9	31.6	284	25.2	3.9	414	200.3	12.01	49.7	IDA	

42	ps 2640/15	4	M	374069	10.8	3.5	8.3	25.7	73.4	22.7	32.3	290	16.6	5.7	404	195	1.71	6.9	IDA	
43	ps 2643/15	1.5	M	374037	7.4	4.7	9.2	29.5	72.5	22.6	31.2	509	20.3	5.8	444	252.8	10.79	47.9	IDA	
44	ps 2675/15	9	F	374207	11.3	3.35	3.9	10.2	54.3	11.6	21.4	886	28.6	1.2	503	267	1.73	8.7	IDA	
45	ps 2691/15	10	M	374235	3.9	1.9	4.8	15.7	79.6	25.3	30.6	264	22.3	4.8	468	189.4	5.96	27.9	IDA	
46	ps 2693/15	1.5	M	374419	8.1	3.3	7.6	24.1	71.7	22.6	31.5	361	19.9	3.2	389	176	13.37	52	IDA	
47	ps 2715/15	3	M	374609	5.9	3.39	7.4	22.8	67.3	21.8	32.5	211	18.7	6.5	402	257	9.46	38	IDA	
48	ps 2739/15	11	F	374826	11.8	3.8	9.9	30.1	79.2	26.1	32.9	830	13.2	102	229	250	21.83	50	ACD	128.8
49	ps 2750/15	5	M	16086	11.9	3.97	9.3	28.6	72	23.4	32.5	315	12.9	4.2	451	290.8	9.10	41	IDA	
50	ps 2756/15	8	F	374890	19.8	3.11	6.8	20.4	65.6	21.9	33.5	463	21.1	2.3	433	280	2.75	11.9	IDA	

MASTER CHART II

Sl.No.	peripheral smear no.	Age & Sex	IP No.	RBC (10^6)	HGB (G/DL)	HCT(%)	MCV (FML)	MCH (pg)	RDW (CV)%	DIAGNOSIS	E&F	mentzer	srivastava	shine &lal	ricerca	sirdah	chsani	RDWI	MDHL	IMCHD	E&F
1	ps 2124/14	3/F	311677	2	3.8	13.9	69.5	19	not measurable	thal	45.1	35	9.5	918		56.1	49.5		0.547	0.273	45
2	ps 2210/14	8/M	314631	5.06	7.7	20.2	57.7	15.2	22	ACD	10.7	11	3.004	506	4.3478	29.54	7.1	250.87	1.333	0.263	11
3	ps 2096/14	4/M	311801	3.46	8.9	28	70.9	25.7	22.6	ACD	19.5	20	7.4277	1292	6.5318	40.74	36.3	463.1	1.254	0.362	20
4	ps 3222/14	12/M	315618	3.72	10.4	32.7	67.9	23	18	IDA	8.78	18	6.1828	1060	4.8387	32.98	30.7	328.55	1.26	0.339	9
5	ps 3310/14	10M/M	315973	4	8	29	74	22	20.8	IDA	26.6	19	5.5	1205	5.2	46	34	384.8	1.189	0.297	27
6	ps 3412/14	7/F	317117	4.04	7.3	29.2	72.3	18.1	24.3	IDA	28.4	18	4.4802	946	6.0149	46.36	31.9	434.87	1.011	0.25	28
7	ps 3509/14	1/M	317673	3.5	4.4	20.1	56.6	12.4	29.8	IDA	27.7	16	3.5429	397	8.5143	39.9	21.6	481.91	0.767	0.219	28
8	ps 3514/14	2/M	323683	4.19	5.8	20.1	56.8	13.7	28	IDA	20.2	14	3.2697	442	6.6826	35.21	14.9	379.57	1.011	0.241	20
9	ps 3520/14	5/M	322765	2.63	4.8	16.8	63.9	18.3	30	IDA	33.9	24	6.9582	747	11.407	46.87	37.6	728.9	0.753	0.286	34
10	ps 3544/14	1/F	336678	3.20	6.3	21.7	66	19.1	23.5	IDA	27.8	20	5.8065	832	7.1429	43.81	33.1	471.43	0.952	0.289	28
11	ps 3569/14	8/F	344535	3.29	5.9	19.8	60.2	17.9	34.9	IDA	24	18	5.44	649	10.608	39.21	27.3	638.6	0.978	0.297	24
12	ps 4906/14	4.5/F	344875	4.27	9.5	33.2	67	22.2	18.8	IDA	11.8	16	5.20	997	4.4028	34.23	24.3	294.99	1.415	0.331	12
13	ps 4907/14	3/M	344856	4.15	9.8	32.5	70.3	23.6	17.9	IDA	13.8	17	5.69	1166	4.3133	36.75	28.8	303.22	1.393	0.336	14
14	ps 4950/14	1/M	344978	3.91	8.3	31.2	79.8	21.2	19.7	IDA	31	20	5.42	1350	5.0384	50.99	40.7	402.06	1.039	0.266	31
15	ps 4978/14	9/M	345090	4.18	7.5	28.8	68.9	17.9	13.3	ACD	23.8	16	4.28	850	3.1818	42.22	27.1	219.23	1.086	0.26	24
16	ps 5708/14	6/F	346246	4.04	8.09	24.5	60.6	19.8	17.8	IDA	12.7	15	4.90	727	4.3069	30.29	20.2	261	1.32	0.327	13
17	ps 5810/14	6M/M	348420	3.58	8.1	29.1	71.3	22.6	16.5	IDA	23.8	20	6.31	1149	4.6089	43.42	35.5	328.62	1.135	0.317	24
18	ps 245/15	2/F	348368	4.08	8.7	30.8	70	21.3	17.2	IDA	19	17	5.22	1044	4.2157	39.82	29.2	295.1	1.241	0.304	19

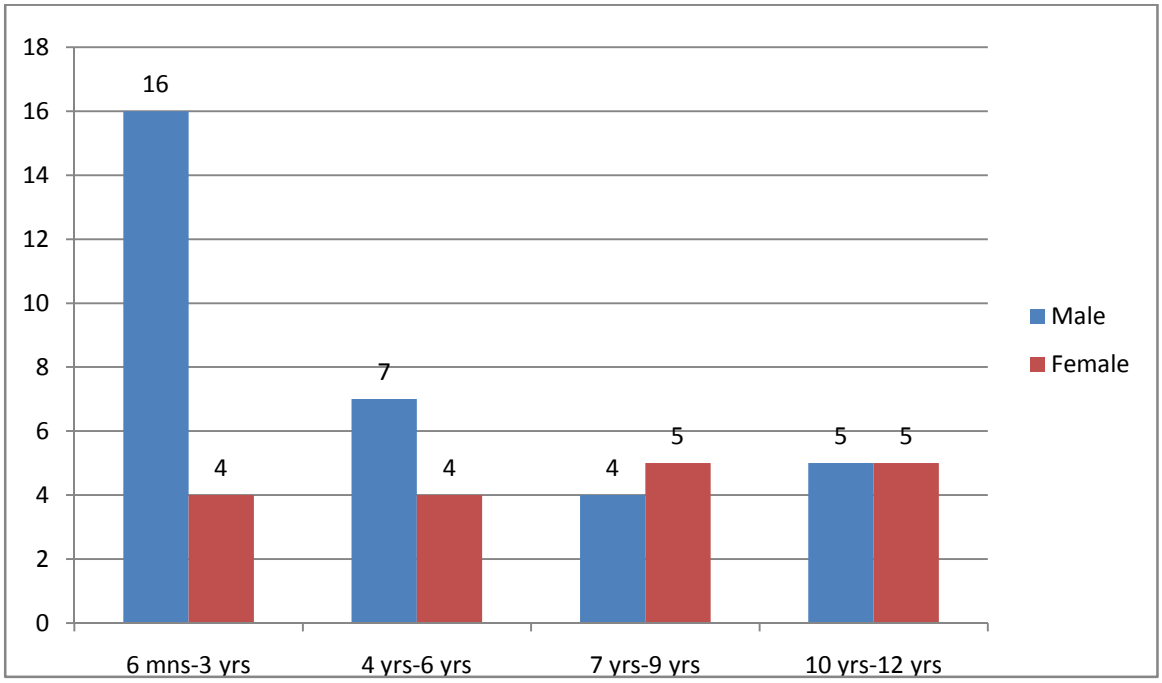
19	ps 364/15	4/M	349176	3.57	4.1	19.8	55.5	11.5	29.7	IDA	28	16	3.22	354	8.3193	39.63	19.8	461.72	0.74	0.207		28
20	ps 1121/15	10/F	345782	3.35	6.5	23	68.7	19.4	12.9	ACD	29.5	21	5.79	916	3.8507	45.85	35.2	264.55	0.946	0.282		29
21	ps 1355/15	2.5/M	356516	4.58	11.4	32.8	71.6	24.9	13.2	ACD	6.62	16	5.44	1277	2.8821	32.82	25.8	206.36	1.593	0.348		7
22	ps 1351/15	6/F	356511	4.68	10	29	76.7	20	12.7	IDA	18.6	16	4.27	1177	2.7137	42.02	29.9	208.14	1.22	0.261		19
23	ps 1444/15	2/M	357474	4.64	6.3	25.2	54.3	13.6	22.2	IDA	14.8	12	2.93	401	4.7845	30.76	7.9	259.8	1.162	0.25		15
24	ps 1574/15	11/M	357713	4.63	7.8	28.3	61.1	16.8	23.1	IDA	18.1	13	3.63	627	4.9897	33.07	14.8	304.84	1.273	0.275		14
25	ps 1698/15	2.5/M	357608	4.75	9	28.1	59.2	18.9	18.8	IDA	6.05	12	3.98	662	3.9579	27.45	11.7	234.31	1.516	0.319		6
26	ps 1549/15	3/M	358584	4.25	10.9	32.9	77.4	25.6	15.5	IDA	15.3	18	6.02	1534	3.6471	40.45	34.9	282.28	1.406	0.331		15
27	ps 1598/15	4.5/M	361574	2.1	6	15.5	73.8	28.6	26.6	IDA	38.3	35	13.62	1558	12.667	53.7	52.8	934.8	0.814	0.388		38
28	ps 2020/15	7/M	362960	3.29	8.6	25.4	77.2	25	19.9	IDA	27.5	23	7.60	1490	6.0486	48.11	44.3	466.95	1.065	0.324		28
29	ps 2121/15	5/M	363546	2.99	4	17.5	58.5	22.9	27.6	IDA	32.1	20	7.66	784	9.2308	43.51	28.6	540	1.17	0.391		32
30	ps 2183/15	11/F	365987	3.95	11.4	31.9	80	25	12.3	ACD	15.7	20	6.33	1600	3.1139	41.85	40.5	249.11	1.234	0.313		16
31	ps 2162/15	12/M	369307	1.91	4.5	15.3	60	23.6	22.3	IDA	52.2	42	12.36	1510	11.675	64.59	60.9	934.03	0.563	0.295		52
32	ps 2175/15	9/F	374207	3.14	3.1	15.8	50.3	22	21.5	IDA	28.3	16	7.01	557	6.8471	37.86	18.9	344.41	1.373	0.437		28
33	ps 2422/15	5/F	372020	4.72	10	28	75	24.8	15.7	IDA	16.9	16	5.25	1395	3.3263	40.28	27.8	249.47	1.561	0.331		17
34	ps 2427/15	3/F	372734	3.6	4.4	13.4	63.8	27.5	25.3	IDA	36.8	40	17.19	1119	15.813	49	47.8	1008.8	0.69	0.431		37
35	ps 2432/15	2/M	372909	3.57	7.3	21.9	69.7	20.4	15.9	IDA	26.2	20	5.71	991	4.4538	44.23	34	310.43	1.015	0.293		26
36	ps 2449/15	6/M	372261	3.8	8.3	25.3	66.6	21.8	17.5	IDA	17.9	18	5.74	967	4.6053	37.9	28.6	306.71	1.244	0.327		18
37	ps 2461/15	10/F	373456	1.58	2.5	9.7	61.4	15.8	27.1	IDA	43.9	39	10.00	596	17.152	52.32	45.6	1053.1	0.407	0.257		44
38	ps 2513/15	1.5/M	372147	3.66	7.2	24.2	66.1	19.7	19.8	IDA	23	18	5.38	861	5.4098	40.84	29.5	357.59	1.091	0.298		23
39	ps 2527/15	1/M	323062	4.51	8.2	27.9	61	18.2	20.8	IDA	12.1	14	4.04	677	4.612	31.89	15.9	281.33	1.346	0.298		12
40	ps 2528/15	9/M	373033	3.47	7.9	27.8	72.6	31.3	15.6	ACD	26.4	21	9.04	1650	5.9193	45.43	37.9	284.34	1.495	0.451		26

41	ps 2565/15	10/F	373423	4.5	9.9	31.3	75.4	23.9	25.2	IDA	18	17	5.31	1359	5.6	41.2	30.4	422.24	1.426	0.317	18
42	ps 2640/15	4/M	374069	3.5	8.3	25.7	73.4	22.7	16.6	IDA	25	21	6.49	1223	4.7429	45	38.4	348.13	1.082	0.309	25
43	ps 2643/15	1.5/M	374037	4.7	9.2	29.5	72.5	22.6	20.3	IDA	18.4	15	4.81	1188	4.3191	40.2	25.5	313.14	1.465	0.312	18
44	ps 2675/15	9/F	374207	3.35	3.9	18.2	54.3	11.6	28.6	IDA	28.1	16	3.46	342	8.5373	39.25	20.8	463.58	0.716	0.214	28
45	ps 2691/15	10/M	374235	1.9	4.8	15.7	79.6	25.3	22.3	IDA	50.3	42	13.32	1603	11.737	63.3	60.6	934.25	0.604	0.318	50
46	ps 2693/15	1.5/M	374419	3.3	7.6	24.1	71.7	22.6	19.9	IDA	27	22	6.85	1162	6.0303	45.6	38.7	432.37	1.04	0.315	27
47	ps 2715/15	3/M	374609	3.39	7.4	22.8	67.3	21.8	18.7	IDA	23.5	20	6.43	987	5.5162	41.71	33.4	371.24	1.098	0.324	24
48	ps 2739/15	11/F	374826	3.8	9.9	30.1	79.2	26.1	13.2	ACD	22.5	21	6.87	1637	3.4737	45.7	41.2	275.12	1.252	0.33	23
49	ps 2750/15	5/M	160886	3.97	9.3	28.6	72	23.4	12.9	IDA	18.1	18	5.89	1213	3.2494	40.13	32.3	233.95	1.29	0.325	18
50	ps 2756/15	8/F	374890	3.11	6.8	20.4	65.6	21.9	21.1	IDA	25.1	21	7.04	942	6.7846	42.09	34.5	445.07	1.038	0.334	25

OBSERVATION AND RESULTS

The age of 50 randomly selected cases in our study ranged from 6 months to 12 years. The age and sex distribution of cases is shown in Diagram 1

DIAGRAM 1

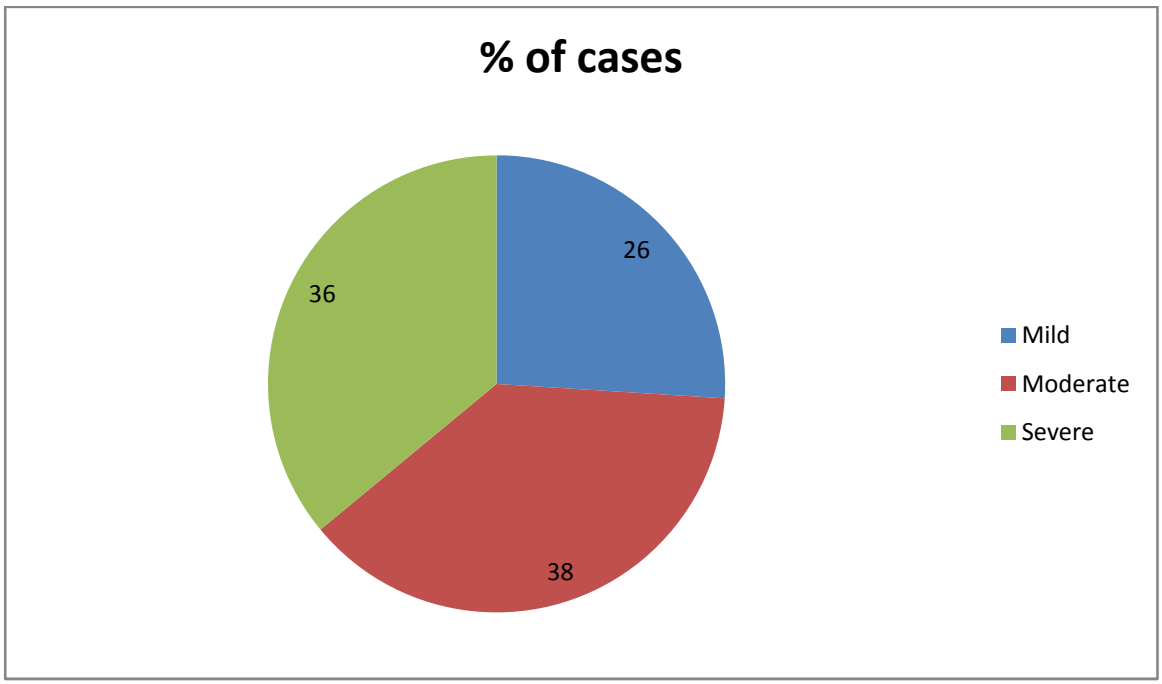


The mean age and median age was 5.64 years. & 5 years respectively.

Among the various clinical features, pica was noticed in 35 cases of IDA (85.3%).

No. of cases with mild, moderate and severe anaemia are 13, 19 & 18 respectively (26%, 38% & 36%). This is shown in Diagram 2

DIAGRAM-2



Ninety nine percentage of cases in our study had RBC count less than 5 million.

Only one case of Thalassemia major showed RBC count greater than 5 million.

Various RBC parameters are shown in the Table 1

TABLE 1

RBC Parameters

	IDA	ACD
RBC count (x 10^6 µL)	3.60 (1.6 – 4.68)	3.9 (3.35 – 5.06)
Hemoglobin (g./dl)	7.19 (3.1 – 10.9)	8.9 (6.5 – 11.4)
Hematocrit (%)	24.22 (9.7 – 33.2)	28.32 (22.8 – 32.8)
MCV (fml)	67.22 (50.3 – 80)	71.2 (57.7 – 79.2)
MCH (pg)	20.67 (11.5 – 28.6)	23.18 (15.2 – 31.3)
MCHC (g/dl)	29.33 (19.6 – 38.7)	29.9 (26 – 34.8)
RDW (%)	21.5 (12.7 – 34.9)	15.38 (12.3 – 22.6)

All the RBC parameters were expressed in mean with the range of values in parenthesis for IDA and ACD cases. Only one case of Thalassemia major was reported in the present study.

The sensitivity, specificity, positive predictive value and negative predictive value of RDW in the diagnosis of IDA was calculated in the following formula.

Sensitivity = (true positive / (true positive + false negative))X100

Specificity = (true negative / (true negative + false positive))X100

PPV = (true positive / (true positive + false positive))X100

NPV = (true negative / (true negative + false negative))X100

The cut off value of RDW for diagnosis of IDA was taken as 13.4 in our study

Test result	**IDA**	**Non-IDA**	**Total**
Positive	39	3	42
Negative	2	6	8

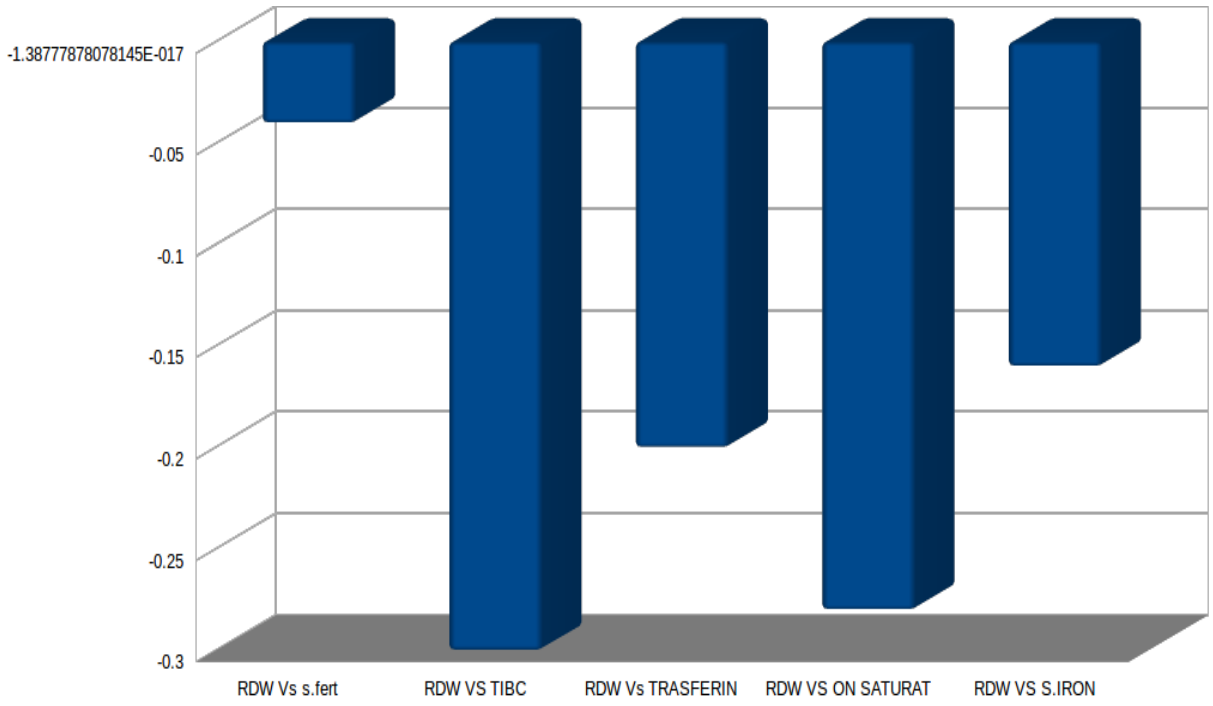
Sensitivity = 39/(39+2) X 100 = 95%

Specificity = 6/(6+3) X 100 = 66%

PPV = 39/(39+3) X 100 = 92.8%

NPV = 6/(6+2) X 100 = 75%

CORRELATION	
RDW Vs S.FERRITIN	r = -0.04
RDW VS TIBC	r = 0.30
RDW Vs TRANSFERRIN	r = -0.20
RDW VS ON SATURATION	r = -0.28
RDW VS S.IRON	r = -0.16



The correlation co-efficient (r) between RDW Vs. Serum Ferritin, RDW Vs. Transferrin, RDW Vs. Percent Saturation of Iron, RDW Vs. S.Iron showed strong negative association, i.e, when one variable increases, other decreases. When the serum ferritin, serum iron, percent saturation increases, the RDW which is a measure of anisocytosis returns to normal values. So this correlation was statistically significant.

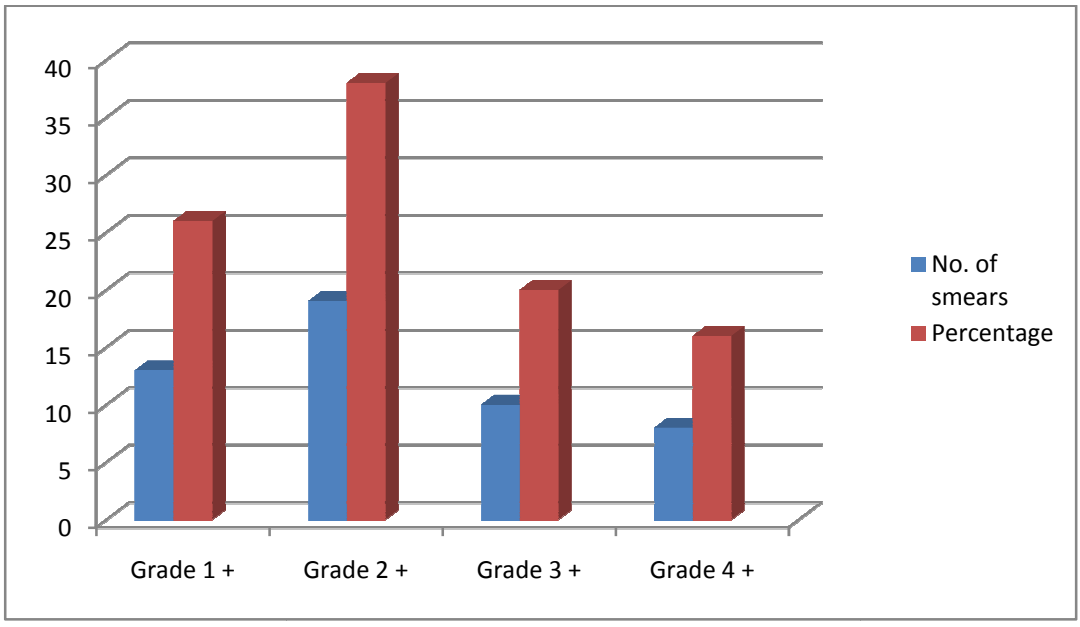
In the Peripheral blood film examination, No. of smears showing pencil cells was 16 (39%) out of 41 cases of IDA, showing that peripheral blood film was sensitive only when the iron deficiency is more pronounced.

Grading of microcytosis and hypochromia was done using Blood cell morphology grading guide by Gene Gulati .

1+ grade	<25 cells/100 RBC'S
2+ grade.	25-50 cells/100 RBC'S.
3+ grade.	50-75 cells/100 RBC'S
4+ grade.	>75 cells/ RBC'S.

In the peripheral blood film, 200 RBC's were counted to check for microcytic and hypochromic cells. In our study, out of 50 cases of anemia, following grade was seen with respect to microcytosis and hypochromia.

	No. of smears	**Percentage**
Grade 1 +	13	26%
Grade 2 +	19	38%
Grade 3 +	10	20%
Grade 4 +	8	16%



The percentage of hypochromic cells according to various studies

The sensitivity, specificity, PPV, NPV and the Youden's index of the various Red cell formulas were calculated and tabulated as follows :

Indices	Cut off values for IDA	No.of cases of IDA correctly diagnosed (n=41)	Percentage
Mentzer's	>13	39	95
Srivastava	>3.8	35	85
Shine & lal	>1530	38	92.6
Ricerca	>4.4	33	80.4
Ehsani	>15	37	90.2
RDWI	>220	40	97.5
MDHL	<1.63	41	100
MCHD	<0.30	13	31.7
Sirdah	>27	41	100
RBC count	<5	41	100
England & Fraser	>0	41	100

The various Red cell formulas, in the order of percentage of IDA cases correctly diagnosed, were ranked as follows:

I. Sirdah, RBC count, E&F, MDHL II. RDWI

III. Mentzer's IV. Shine & Lal

V. Ehsani VI. Srivatsava

VII. Ricerca VIII. MCHD

Indices	Sensitivity	Specificity	PPV	NPV	Youden's
Mentzer's	95.12	11.11	82.97	33.33	6.23
Srivastava	85.36	11.11	81.39	14.28	3.52
Shine & lal	7.31	6.66	50	13.63	-26.1
Ricerca	8.48	77.77	94.28	46.66	58.26
Ehsani	9.24	11.11	82.22	20	1.35
RDWI	97.56	22.22	85.10	66.66	19.78
MDHL	100	0	82	-	0
MCHD	31.70	55.55	76.47	15.15	-12.73
Sirdah	100	0	82	-	0
RBC count	100	11.11	83.67	100	11.11
England & Fraser	100	0	82	-	0

Highest sensitivity was ranked in the following order:

I. Sirdah, RBC count, E&F, MDHL II. RDWI >

III. Mentzer's IV. Srivastava

V. MCHD VI. Ehsani

VII. Ricerca VIII. Shine & Lal

Highest specificity was ranked in the following order:

I. Ricerca II. MCHD

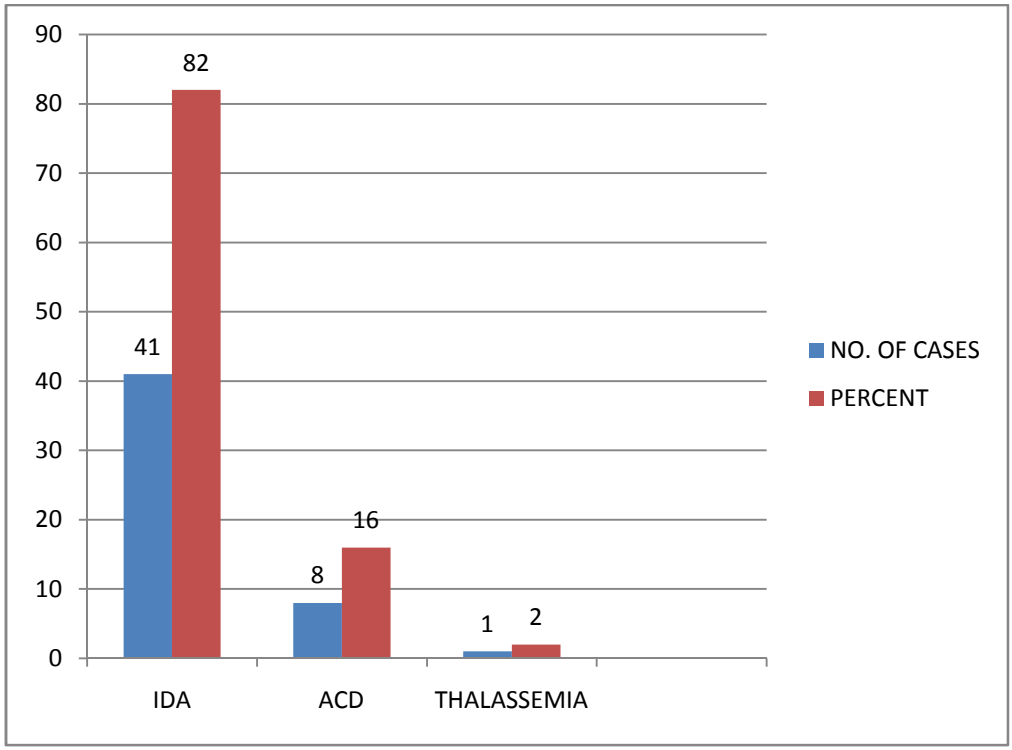
Highest PPV was ranked in the following order:

I. Ricerca II. RDWI

Highest NPV was shown by RDWI

Youden's index was highest for Ricerca index.

TYPE OF CASE	NO OF CASES	PERCENTAGE
IDA	41	82 %
ACD	8	16 %
THALASSEMIA MAJOR.	1	2 %
TOTAL	**50**	**100 %**
AVERAGE	**16.67**	



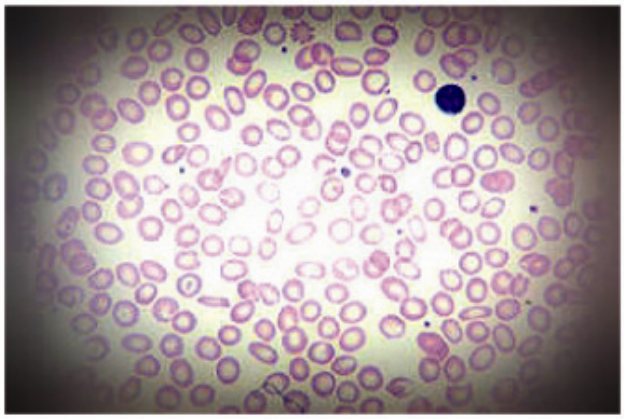


Fig.1. Thalassemia Major- Peripheral blood film showing marked anisopoikilocytosis with severe microcytic hypochromic red blood cells-low power view.

Fig . 2. Thalassemia Major- Peripheral blood film showing tear drop cells and target cells- High power view.a

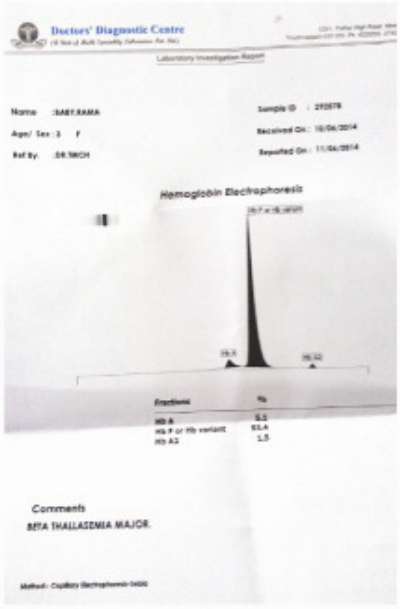


Fig .3. Thalassemia Major- Hemoglobin Electrophoresis shows elevated HbF fraction confirmatory of diagnosis.

Fig .4. Thalassemia Major- Mongolian like facies with flattened nasal bridge and prominent malar bones.

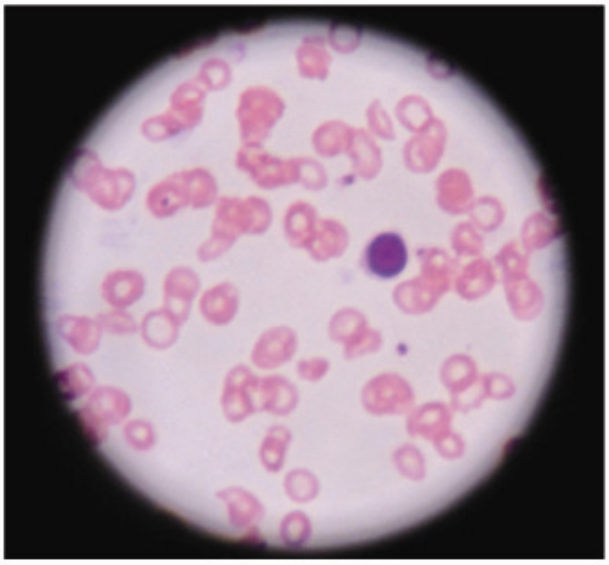


Fig .5. Iron deficiency anemia- peripheral blood film showing microcytosis (compared with the size of small lymphocyte) and hypochromia.

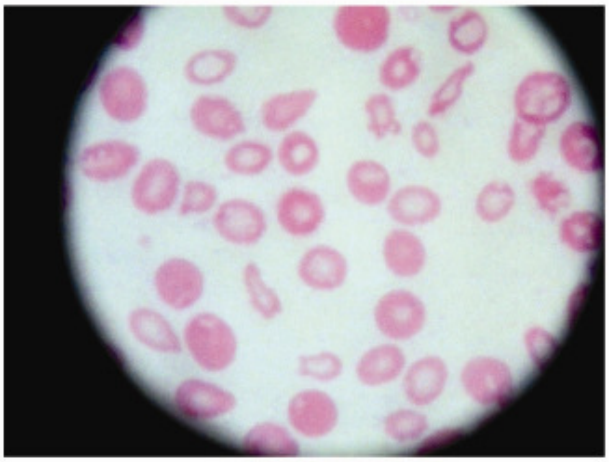


Fig .6. Iron deficiency anemia- peripheral blood film-High Power view showing two pencil cells characteristic of this anemia.

Fig .7. SYSMEX KX-21 HEMATOLOGY ANALYSER

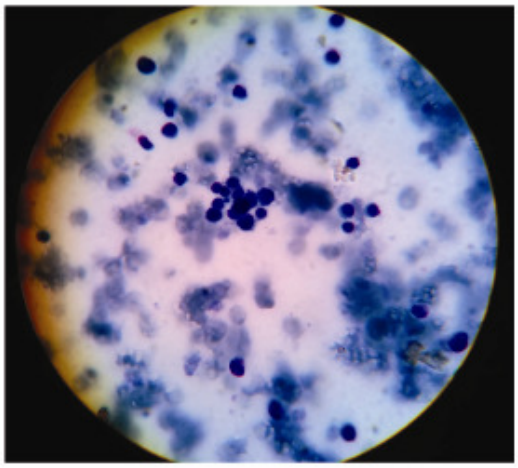


Fig .8. Bone Marrow Aspirate in Iron deficiency Anemia- low power view of leishman stained smear showing colonies of micronormoblasts.

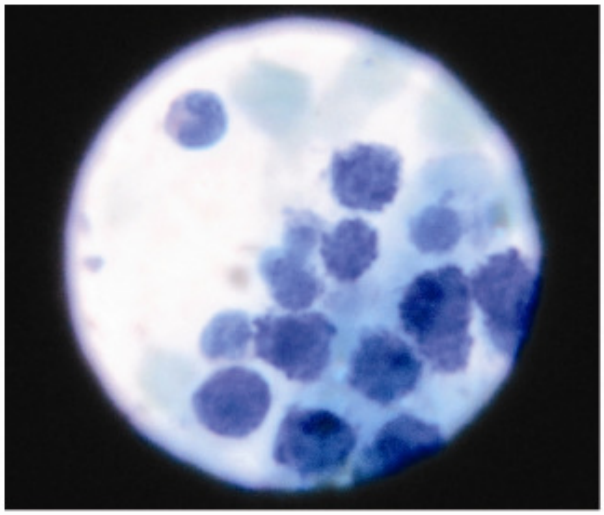


Fig. 9. Bone Marrow Aspirate in Iron Deficiency Anemia-High Power View of leishman stained smear showing frayed cytoplasmic borders and persistent basophilia in the micronormoblasts.

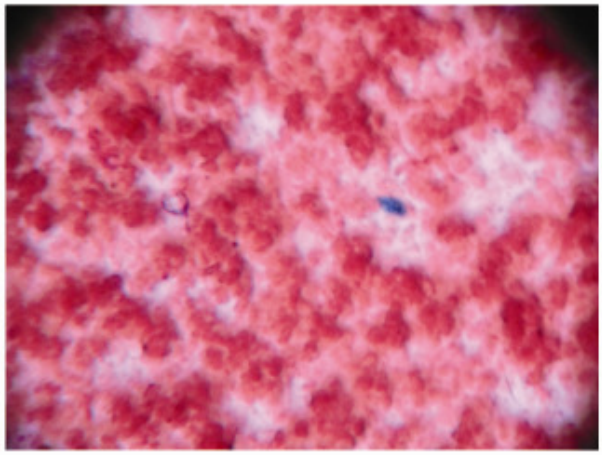


Fig. 10. Bone Marrow Aspirate- Prussian Blue Stain showing absent iron stores in Iron Deficiency Anemia.

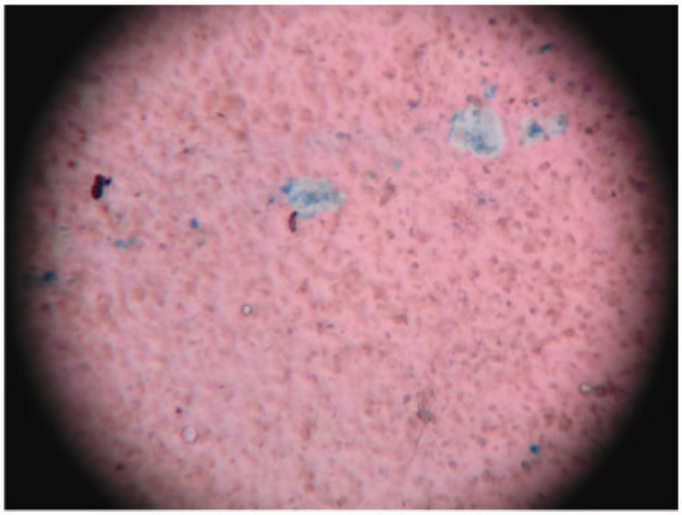


Fig .11. Bone Marrow Aspirate- Prussian Blue Stain with mildly increased iron stores (grade 3+) in Anemia of chronic disease.

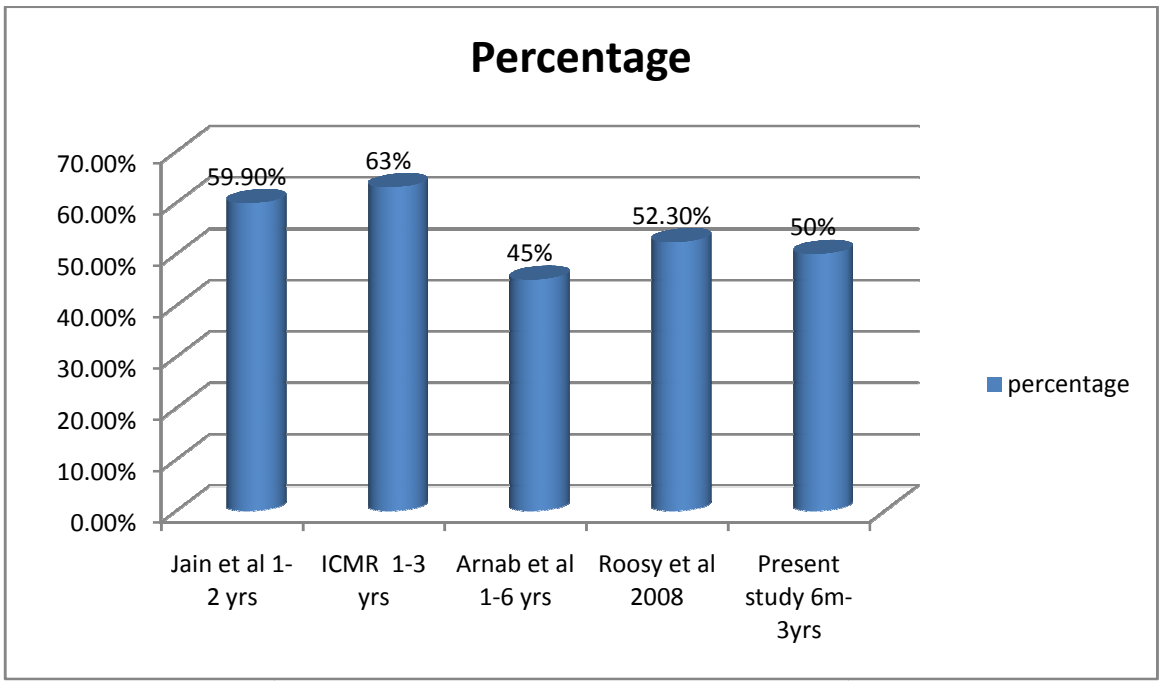
DISCUSSION

In our present study involving 50 randomly selected cases, the various red cell indices like hemoglobin, RBC count ,MCV, MCH and MCHC were obtained from the SYSMEX 3 part analyser. The Serum iron profile which comprised of serum iron, serum ferritin, Total iron binding capacity and percent saturation of transferring was obtained from a private laboratory with specified reference values. Assuming, the serum iron profile to be more helpful as a gold standard in the differential diagnosis of microcytic hypochromic anemia, the various parameters such as RBC count, Hemoglobin, RDW were correlated with iron profile .

In our present study, the predominant age group involved was 6 months to 3 years.(50%).In their study, Jain et al 2000[13] have reported high prevalence of anemia between 1 to 2 years(59.9%).In the ICMR Study 1985[13], age group most involved was also between 1 to 3 years.(63%). The hospital based study in Nepal by Arnab et al[10] showed commonest age group between 1-6 years.[10]. Roosy Aulakh et al[14] , in their study reported increased prevalence in the age group between 1-5 years.

Table No: 1

study	Age group	percentage
Jain et al 2000	1-2 years	59.9%
ICMR 1985	1-3 years	63%
Arnab et al2015	1-6 years	45%
Roosy Aulakh et al 2008	1-5 years	52.3%
Present study 2015	6 months-3 years	50%



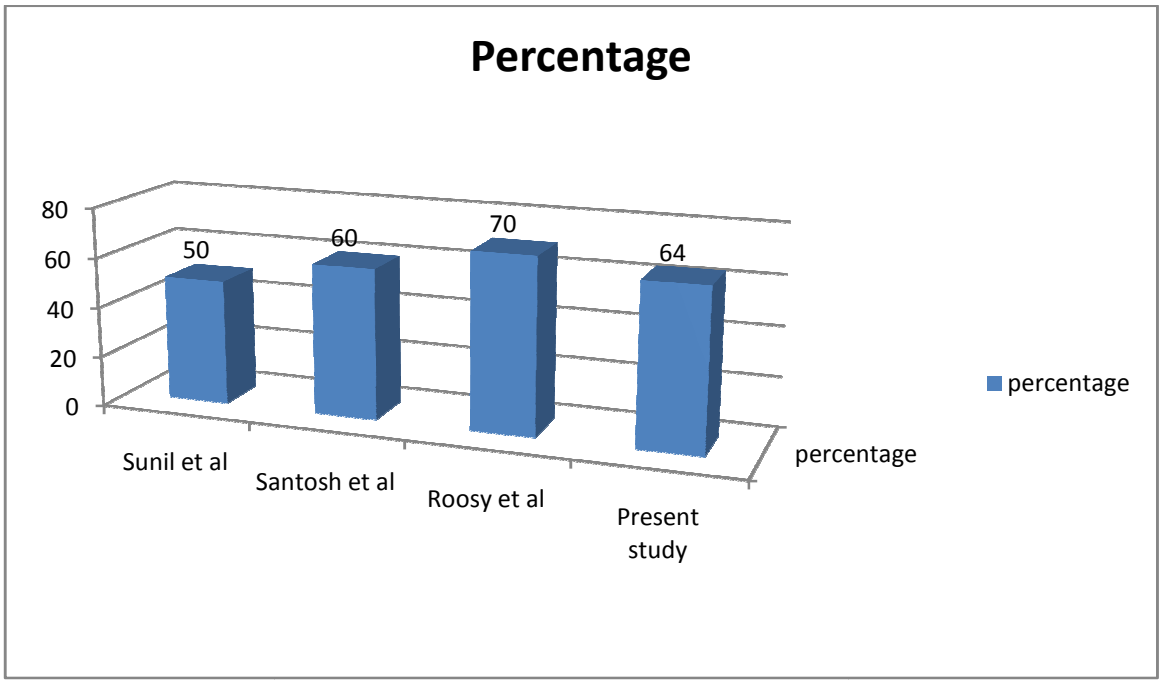
Our study corresponds to the other studies in terms of predominant age group involved.ie.more number of children with anemia are found in the age group between 6 months to 3 years.

In terms of predominant sex involved, present study showed out of 50 children more males (32)(64%)were involved than females (18)(36%). This corresponded to other studies also.Sunil et al[5] found a 50% male children involved in their study. Santosh

et al [13] and Roosy Aulakh et al[14] have reported a 60% and 70% of male involvement in their studies.

Table No:2

Sunil et al 2014	50%
Santosh et al 2013	60%
Roosy et al 2008	70%
Present study 2015	64%



The mean values of various RBC parameters like mean Hb ,mean MCH, mean MCV, mean RDW and the biochemical profile like mean serum ferritin in our study were in coincidence with the other studies like Sazawal et al 2014[5] and Santosh et al 2013[13]. This is shown in the following table:

Table No:3 Comparison with Sazawal et al

	Present study		Sazawal et al	
Parameters	**Iron deficient anemia.**	**Non iron deficient anemia.**	**Iron deficient anemia.**	**Non iron deficient anemia.**
Mean RBC(millions)	**3.6**	**3.9**	**4.2**	**4.4**
Mean Hb(g/dl)	**7.19**	**8.9**	**8.4**	**10.9**
Mean MCV(fml)	**67.22**	**71.2**	**69.6**	**78.9**
Mean MCH(Pg)	**20.67**	**23.18**	**20.0**	**24.2**
Mean RDW(%)	**21.57**	**15.38**	**19.9**	**16.8**
Mean Ferritin	**8.24 ng/ml**	**233.3 ng/ml**	**6.2 µg/l**	**16.3 µg/l**

Table No:4: Comparison with Santosh et al

	Present study		Santosh et al	
Parameters	**Iron deficient anemia.**	**Non iron deficient anemia.**	**Iron deficient anemia.**	**Non iron deficient anemia.**
Mean RBC(millions)	**3.6**	**3.9**	**4.01**	**4.4**
Mean Hb (g/dl)	**7.19**	**8.9**	**9.0**	**10.9**
Mean MCV(fml)	**67.22**	**71.2**	**70.3**	**78.9**
Mean MCH(Pg)	**20.67**	**23.18**	**23.3**	**24.2**
Mean RDW(%)	**21.57**	**15.38**	**16.6**	**16.8**
Mean Ferritin	**8.24ng/ml**	**233.3ng/ml**	**6.2µg/l**	**16.3 µg/l**

These parameters are compared to show that the mean values derieved in our study and from the other studies were not significantly different to affect the study outcome. The serum ferritin value alone showed difference because the reference values of individual laboratories vary between each other. In our study, the laboratory reference value was between 7-140 ng/ml.

The sensitivity and specificity of Red cell distribution width (RDW) was compared with that of other studies. Various studies reported more than 95% to 100 % sensitivity in the diagnosis of early iron deficiency. In the present study, the sensitivity and specificity was 95% and 66% respectively. The following table shows the comparison with other studies.

Study	Sensitivity and specificity(%)	Cut off value in %
Santosh et al 2013	100	-
Viswanath et al 2001 and England et al 1976.	100	-
Mc Clure et al 1985	100	-
Bessmann et al 1983	96	-
Von Zeben et al 1990	94 & 59	14.5
Flynn et al 1998	94 & 51	13.4
Kim et al 1996	83 & 57	15
Aulakh et al 2008	81 & 53	17.4
Sazawal et al 2014	90.4 & 98.9	15
Present study 2015	95 & 66	13.4

From the above table it is obvious that as the cut off value is lowered from 17.4 to 13.4, the sensitivity increases but the specificity decreases. In the present study, the specificity of 66% could be attributed to the low cut off value. But in our country where IDA is the more prevalent microcytic hypochromic anemia, a low cut off value is permissible as it could pick up more cases of IDA which is advantageous in term of long term outcome. But the disadvantage is that when the specificity is lost, more cases of anemia of chronic diseases and some cases of heterozygous α and β thalassemias, where RDW is normal would also be included in the list of IDA. So as a reasonable balance, the cut off value of RDW would be set as >15% as in the

study of Sazawal et al and Kim et al[5], which would allow a good sensitivity and specificity.

According to Sezawal et al[5], the expensive investigations needed for the diagnosis of most common microcytic anemia,IDA such as iron status markers necessitates the use of cost effective tools. The increase in RDW may occur even before the decrease in MCV. They also conclude that RDW may very well be employed in large sample settings as a screening tool to identify iron deficiency anemia. They suggest a cut off value of RDW >15% and hemoglobin <10 g/dl.

As seen in the observation and results, there was a statistically significant negative association between RDW and serum ferritin, serum transferrin, percent iron saturation.

Although the various red cell formulas are used to discriminate IDA from Beta thalassemia especially thalassemia trait(discriminant index),Sazawal et al[5] suggest that they can still be sued to predict IDA

1. Mentzer index:

   In our study the percentage of correctly diagnosed children in the case of IDA was 95% which conforms to studies of Aysel et al[4] (91%) and Ehsani et al (94%). The sensitivity (95%) was also in concordance with the above studies .

Study	Sensitivity	specificity
Aysel et al	98.7%	82.3%
Ehsani et al	95.5%	94.6%
Present study	95.12%	11.11%

The high sensitivity means the index is able to predict more IDA cases. But the low specificity in the present study was due to the control group of anemia cases used, which were mostly ACD cases. In contrast, in the above studies, β-Thalassemia trait cases were used as controls, as those studies were conducted in thalassemia belts with high prevalence of thalassemia trait cases. The formula uses RBC count as the denominator. In the study by Aysel et al, the mean RBC count was 5.6+ or -4. In our study the mean RBC count was 3.6 million. In thalassemia trait the RBC count is normal or even increased[20]. With the high RBC counts the Mentzer's index would be less than 13 resulting in the high specificity in their study. In the present study, majority of ACD cases(88%) had less RBC counts that had resulted in Mentzer's index greater than 13 and thus low specificity. More over, in the present study, all the ACD cases had a higher CRP values which was predictive of anemia of chronic disease.

Srivastava (1973) index:

Here again, the formula uses RBC count as the denominator, but the cut off to diagnose IDA was greater than 3.8. So the sensitivity in their study Aysel et al [4],was about 72% and the specificity was 85%. In our study, the sensitivity and specificity were 85.36% and 11.11% respectively.

The following table compares the sensitivity and specificity of the other indices with the corresponding values from the other 2 studies.

Indices	Sensitivity	Specificity
Srivastava		
Present study	85.36	11.11
Aysel et al	72	85.7
Ehsani et al	88.5	85.7

Indices	Sensitivity	Specificity
Shine & lal		
Present study	7.31	6.66
Aysel et al	10.2	100
Ehsani et al	-	-

Indices	Sensitivity	Specificity
Ricerca		
Present study	8.48	77.77
Aysel et al	14.7	100
Ehsani et al	-	-

Indices	Sensitivity	Specificity
Ehsani		
Present study	9.24	11.11
Aysel et al	73.5	94.8
Ehsani et al	90	95.5

Indices	Sensitivity	Specificity
RDWI		
Present study	97.56	22.22
Aysel et al	76.4	83.1
Ehsani et al	-	-

Indices	Sensitivity	Specificity
MDHL		
Present study	100	0
Aysel et al	58.8	76
Ehsani et al	-	-

Indices	Sensitivity	Specificity
MCHD		
Present study	31.70	55.55
Aysel et al	27.9	77.9
Ehsani et al	-	-

Indices	Sensitivity	Specificity
Sirdah		
Present study	100	0
Aysel et al	22	108
Ehsani et al	-	-

Indices	Sensitivity	Specificity
RBC count		
Present study	100	11.11
Aysel et al	8	96
Ehsani et al	86.2	98.1

Indices	Sensitivity	Specificity
England & Fraser		
Present study	100	0
Aysel et al	52	116
Ehsani et al	99.2	69.5

In the present study, the best indices to predict IDA were in the following order:

1. Sirdah.

2. RBC count.

3. E&F.

4. Mean density of hemoglobin per liter of blood (MDHL).

5. RDWI.

6. Mentzer's index.

This is in contrast to Aysel et al who found in their study Mentzer index to be most reliable and Ehsani et al who found that Mentzer to be the most reliable and then Ehsani to be the second most sensitive index to discriminate between IDA and β TT.

In the present study the indices with the highest sensitivity were Sirdah ,RBC count, England and Fraser index. The following table shows comparison with other studies:

Studies	Sample size	Best index
Demir et al(2002)	63	RBC Count
Ntaios et al(2007)	493	Green & King
Beyan et al(2007)	111	RBC count
Urrechaga et al(2008)	2196	Green & King
Sirdah et al(2008)	2196	Green & King
Present study(2015)	50	Sirdah ,E & F RBC count.

It is important to that iron deficiency anemia is treated adequately in young children. Often the clinical history, diet enquiry ,physical examination of the child and the peripheral blood film along with the measurement of red cell indices leads to the correct diagnosis. In case of doubt , a therapeutic trial of iron may be given with dietary advice. This approach is reasonable without any harm even in case of infections or a thalassemia trait. The investigation of thalassemia trait may be done after correction of iron deficiency. If the hemoglobin concentration and red cell indices do not return to normal, then further investigation may be done [(9)].

CONCLUSION

1. Microcytic hypochromic anemia is mostly caused by iron deficiency, thalassemias, anemia of chronic disease and very rarely sideroblastic anemia. In the present study, Iron deficiency anemia and anemia of chronic disease were found most commonly than thalassemia or sideroblastic anemia.

2. The basic hematology data obtained from a automated hematology analyser such as MCV and MCH are helpful in classifying anemia into microcytic hypochromic or other categories.

3. The RDW, which is a measure of anisocytosis is an early sign of iron deficiency. The changes in RDW occur early than MCV changes. In the present study, the RDW was found to be very sensitive but less specific in the early detection of iron deficiency.

4. There was also significant negative association for RDW with serum ferritin, serum transferrin, serum iron values which shows that with increase in the iron values after treatment, the RDW returns to normal. The association is therefore statistically significant.

5. The various red cell formulas calculated in the present study showed that the indices sirdah, RDWI, England and Fraser were 100 % sensitive but less specific. The low specificity was attributable to the low RBC count in the present study.

6. The C-Reactive protein was also useful in distinguishing between the iron deficiency and anemia of chronic disease cases in the present study.

7. The peripheral smear was useful only in severe cases of iron deficiency with the identification of pencil cells but overall the sensitivity was very low in mild and moderate iron deficiency.

8. In the present study there was only one case of thalassemia major which was clinically identified by the typical facies, abundance of target cells in the peripheral blood film and the typical hemoglobin electrophoresis pattern.

9. Therefore in the Iron deficient anemias, the recommended cost effective strategy with the presently available facilities in a low resource setting would be.

a) A low MCV and increased RDW with a cut off value of <15 in the highly prevalent areas to pick up more cases with increased sensitivity.

b) The peripheral smear may be used to confirm the morphology as microcytic hypochromic and the presence of pencil cells in severe cases.

c) The calculation of various red cell indices could be easily made to detect iron deficient anemia.

d) Lastly a reticulocyte count could be done not only to rule out hemolysis but also to assess the bone marrow response after a trial of oral iron therapy after 2 weeks. A CRP measurement may be done in all microcytic anemias to rule out as well as diagnose anemia of chronic disease.

10. The strategy in case of anemia of chronic disease would be as follows:

a) The clinical manifestations of the underlying infections and inflammatory conditions. In our study many children had juvenile rheumatoid arthritis and few were diagnosed as to have renal disease.

b) Increased Erythrocyte sedimentation Rate (ESR) and CRP values.

c) Other investigations directed towards the diagnosis of underlying inflammatory condition and treatment. With treatment, a peripheral smear and Complete hemogram may be done to check for correction of anemia. If no correction of anemia is seen, only then costly investigations such as serum hepcidin and serum iron markers may be done.

11.In case of thalassemias, the diagnosis and confirmation of thalassemia major depends heavily on costly strategies such as hemoglobin electrophoresis which is justifiable with the morbidity of the disease namely life long blood transfusion programmes.

12. The cases of thalassemia minor or trait may be initially screened by a elevated RBC count and a normal RDW value along with the use of discriminant red cell indices but still newer cost effective strategies need to be designed.

APPENDIX- 1

TABLE 1:

Hematological values for normal children (expressed as mean ± 2 SD or 95% range)

	1 year	2-6 years	6-12 years
Red cell count(10^{12}/l)	4.5 + 0.6	4.6 + 0.6	4.6 + 0.6
Hemoglobin concentration (g/l)	126 + 15	125 + 15	135 + 20
Hematocrit (Hct) or packed cell volume(PCV) (l/l)	0.34 + 0.04	0.37 + 0.03	0.40 + 0.05
Mean cell volume (MCV) (fml)	78 + 6	81 + 6	86 + 9
Mean cell hemoglobin (MCH) (pg)	27 + 2	27 + 3	29 + 4
Mean cell hemoglobin concentration (MCHC) (g/l)	340 + 20	340 +30	340 + 30
Reticulocytes (10^9/l)	30 -100	30 -100	30 -100
Platelets (10^9/l)	200 -550	200 -490	170 -450

APPENDIX -2

Preparation of leishman's stain and staining method:

1. Weigh 200 mg of the powdered dye.

2. Transfer the content to a conical flask of 200 -250 ml capacity.

3. Add 100 ml of methanol.

4. Warm the mixture to 50* C for 15 min with occasional shaking.

5. Allow the flask to cool and filter the solution.

Staining method:

1. Air dry the blood film.

2. Flood the slide with the leishman's stain.

3. After 2 min, add double the volume of water and stain for 5-7 min.

4. Wash in a stream of buffered water with pH 6.8 until a pinkish tinge appears.

5. Wipe the back of the slide and stand the slide upright to dry.

APPENDIX-3

Reticulocyte Stain and Reticulocyte count:

New Methylene Blue is the commonly used stain for reticulocyte count . It gives better results than brilliant cresyl blue because it stains the reticulo filamentous material more deeply and more uniformly than does brilliant cresyl blue.

1. Dissolve 1 g of New Methylene blue in 100 ml of 3 % trisodium citrate solution.

2. Filter once the dye has been dissolved.

Method:

1.Deliver 2 to 3 drops of the dye solution into 1 75 x 10 mm plastic tube with a Pasteur pipette.

2. Add 2 to 4 volumes of the patient's EDTA anticoagulated blood to the dye solution and mix.

3. Keep the mixture at 37 * C for 15 -20 min.

4. Resuspend the red cells by gentle mixing of the solution.

5. Make films on the glass slides in the same manner as blood film.

6. When dry, examine the films without fixing or counter staining.

Result:

The reticulofilamentous material is stained deep blue. The non reticulated cells are stained in diffuse shades of pale greenish blue.

APPENDIX 4

Prussian Blue stain and staining method of bone marrow aspirate:

1. Mix equal volumes of 20 g/l potassium ferrocyanide and 0.2 mmol/l of Hcl.

Method:

1. Air dry film containing bone marrow aspirate.
2. Fix with methanol for 10 -20 min.
3. After drying, place the slides in a solution containing 10 g/l potassium ferrocyanide in 0.1 mol/l of HCl.
4. Leave the slides in the solution for about 10 min at about 20*C.
5. Wash well in running tap water for about 20 min.
6. Rinse thoroughly in distilled water.
7. Counter stain with 1g/l aqueous eosin for 10 – 15 sec.
8. Wash in running water and stand upright to dry.

Precautions:

1. Avoid contamination by iron present on the slides or dishes.
2. Prepare the glass ware by soaking in 3 mol/l HCL before washing.
3. A positive bone marrow film is stained together with the test films for quality control.

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